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# THE CHOLINERGIC ANTI-INFLAMMATORY PATHWAY AND HEART RATE VARIABILTY WITH SPECIAL REFERENCE TO DIALYSIS AND RENAL DENERVATION

Marie Hilderman


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Enter Year

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# THE CHOLINERGIC ANTI-INFLAMMATORY PATHWAY AND HEART RATE VARIABILTY WITH SPECIAL REFERENCE TO DIALYSIS AND RENAL DENERVATION

## THESIS FOR DOCTORAL DEGREE (Ph.D.)

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# ABSTRACT

**Background**: The Cholinergic Anti-inflammatory Pathway (CAP) is a nerve-mediated circuit through which the vagus nerve regulates the activity of the inflammatory immune response. Injury, ischemia or infection activate the afferent vagus. In the medulla oblongata. a response is subsequently returned via the efferent vagus, referred to as the inflammatory reflex. The signal is propagated to the spleen where specialized T-cells (ChAT) synthesize acetylcholine that binds to α-7-nicotinic acetylcholine receptors on macrophages leading to a down-regulation of inflammatory cytokines. CAP is modified with drugs or by vagus nerve stimulation (VNS) in animal models of sepsis, rheumatoid arthritis (RA) and inflammatory bowel disease (IBD). Clinical VNS pilot studies have demonstrated reduced inflammation and improvement in clinical symptoms in RA and IBD. Chronic kidney disease (CKD), characterized by autonomic dysfunction (AD), is linked to chronic inflammation and associated with excessive morbidity and mortality mainly from cardiovascular disease.

**The aims** of this thesis were to study CAP and VNS in dialysis patients, and to investigate if renal denervation (RDN), in patients with resistant hypertension, could influence CAP. A final objective was to identify the optimal length of ECG for heart rate variability (HRV), a noninvasive tool to assess the cardiac autonomic nervous system, and correlates to inflammatory markers.

**Methods and subjects**: In study I-III we analyzed LPS stimulated cytokine levels ex vivo and the effect of GTS-21, a cholinergic analogue.

**Study I**. Twenty dialysis patients and 8 healthy controls provided blood samples which were analyzed in the LPS-model. ECG was recorded for HRV in some patients and in all controls.

**Study II**. Ten patients scheduled for RDN and 4 disease controls (DC) for elective coronary angiography (CA) were included. Bloods were drawn and ECG recorded before procedures, 1 day after, and in RND patients at follow-up after 3 and 6 months.

**Study III**. Twelve dialysis patients underwent VNS treatment with a minimally invasive oscillating device before dialysis for 4 weeks. Bloods were drawn and ECG was recorded at baseline, after 2 and 4 weeks of treatment, and at 8- and 12-weeks follow-up.

**Study IV**. The database HeartBEAT contains inflammatory markers and nocturnal ECG recordings from 318 subjects at baseline and 301 at follow-up. The data was used to analyze correlations between inflammatory markers, HRV indices, and different lengths of ECG recording.

**Results and conclusion**: In study I we found that the LPS-induced cytokine response was stronger in patients, where reduced HRV confirmed autonomic dysfunvtion, than in controls. Adding GTS-21 decreased cytokines in both groups, suggesting that dialysis patients also have a functional CAP. Study II showed that RDN had a strong effect on inflammatory markers 1 day after RDN, which disappeared during follow-up. With VNS in hemodialysis patients (study III) trends in cytokine response appeared but did not reach statistical significance. There is a negative correlation between inflammatory markers and HRV indices, but CRP alone is not a reliable predictor of autonomic dysfunction. ECG recording length of 60 minutes secured reliable HRV analysis. Outside of the protocol, in study III, we

found that 3 out of 4 diabetics could reduce insulin doses with 25 % during the study suggesting a potential benefit regarding insulin resistance. Furthermore GTS-21 strengthened the cytokine response in study III suggesting a potential additional capacity for VNS. Dialysis and CKD patients may be suitable for non-invasive VNS with daily or prolonged treatment in future trials. It appears as longer ECG recordings may be useful, but HRV as prognostic tool for anti-inflammatory interventions require further studies.

# LIST OF SCIENTIFIC PAPERS

I. **HILDERMAN M**, Qureshi AR, Al-Abed Y, Abtahi F, Lindecrantz K, Anderstam B, Bruchfeld A
Cholinergic anti-inflammatory pathway activity in dialysis patients: a role for neuroimmunomodulation?
*Clinical Kidney Journal*. 2015;8(5):599–605

II. **HILDERMAN M**, Qureshi AR, Abtahi F, Witt N, Jägren C, Olbers J, Delle M, Lindecrantz K, Annette Bruchfeld A
The cholinergic anti-inflammatory pathway in resistant hypertension treated with renal denervation
*Molecular Medicine.* 2019 (25), Article number: 39

III. **HILDERMAN M,** Qureshi AR, Abtahi F, Lindecrantz K, Bruchfeld A
Minimally Invasive Vagus Nerve Stimulation in Hemodialysis Patients and Inflammatory Response – a pilot study
*Manuscript*

IV. Abtahi F, **HILDERMAN M**, Bruchfeld A, Seoane F, Janerot-Sjoberg B and Lindecrantz K
Prognostic Potential of Heart Rate and Heart Rate Variability for Monitoring the Cholinergic Anti-inflammatory Pathway
*Manuscript*

# CONTENTS

# LIST OF ABBREVIATIONS

| | |
|---|---|
| Ach | Acetylcholine |
| AKD | Acute kidney disease |
| AKI | Acute kidney injury |
| Ang II | Angiotensin II |
| ANS | Autonomic nervous system |
| APD | Automated peritoneal dialysis |
| BP | Blood Pressure |
| BPM | Beats per minute |
| CA | Coronary angiogrphy |
| CAA | Cholinesterase activity |
| CAP | Cholinergic anti-inflammatory pathway |
| CAPD | Continuous ambulatory peritoneal dialysis |
| CKD | Chronic kidney disease |
| CNS | Central nervous system |
| CPAP | Continuous positive airway pressure |
| CRP | C-reactive protein |
| CVD | Cardiovaskular disease |
| DAMP | Damage-associated molecular pattern |
| DC | Disease control |
| DMN | Dorsal motor nucleus |
| DOCA | Deoxycorticosteroneacetate |
| DPB | Diastolic blood pressure |
| ECG | Electrocardiogram |
| eGFR | Estimated glomerular filtration rate |
| EHT | Essential hypertension |
| ELISA | Enzyme-linked immunosorbent assay |
| EPO | Erythropoietin |
| ESC | European Society of Cardiology |
| ESH | European Society of Hypertension |
| ESRD | End stage renal disease |
| F | French |
| FDA | Food and Drug Administration |
| GFR | Glomerular filtration rate |
| GTS 21 | i.e. DMXB-A: 3,2,4-dimethoxybenzylidene anabaseine |
| Hb | Hemoglobin |
| HD | Hemodialysis |
| HeartBEAT | Heart Biomarker Evaluation in Apnea Treatment |
| HF | High frequency |
| HIV | Human immunodeficiency virus |
| HLSE | Healthy lifestyle and sleep education |
| HMGB1 | High mobility group B1 |

| | |
|---|---|
| HR | Heart rate |
| HRV | Heart rate variability |
| hsCRP | High sensitive C-reactive protein |
| i.v. | Intravenous |
| IBD | Inflammatory bowel disease |
| IL | Interleukin |
| KDIGO | Kidney Disease: Improving Global Outcomes |
| LF | Low frequency |
| LPS | Lipopolysaccharide |
| MNSA | Muscle sympathetic nerve activity |
| Mϕ | Macrophage |
| NA | Nucleus Ambigus |
| NE | Norepinephrine |
| NN | Normal RR intervals |
| NN50 | Number of adjacent NN-interval > 50 ms |
| NSRR | National Sleep Research Resource |
| o.d. | Oral daily |
| OSA | Obstructive sleep apnea |
| P- | Plasma |
| PAMP | Pathogen-associated molecular pattern |
| PBS | Phosphate buffered saline |
| PCI | Percutaneous coronary intervention |
| PD | Peritonealdialysis |
| pNN50 | Percent of differences between adjacent NN > 50 ms |
| PNS | Parasympathetic nervous system |
| PRR | Pattern recognition receptor |
| RA | Rheumatoid Arthritis |
| RAAS | Renin angiotensin aldosterone system |
| RDN | Renal denervation |
| RMSSD | The square root of the mean of the sum of the squares of differences between adjacent NN |
| RRT | Renal replacement therapy |
| S- | Serum |
| SDANN | Standard deviation of the average NN in 5 min epochs |
| SDNN | Standard deviation of NN |
| SLE | Systemic Lupus Erythematosus |
| SNS | Sympatheitc nervous system |
| SPB | Systolic blood pressure |
| SRR | Swedish Renal Registry |
| T2DM | Type 2 Diabetes Mellitus |
| T1DM | Type 1 Diabetes Mellitus |
| TBI | Thoracic bioimpedance |

| | |
|---|---|
| TLR | Toll-like receptor |
| TNF | Tumor necrosis factor |
| tVNS | Transcutaneous vagus nerve stimulation |
| WHO | World Health Organization |
| VLF | Very low frequency |
| VNS | Vagus nerve stimulation |
| WBC | White blood cell count |
| α7nAChR | α-7-nicotinic acetylcholine receptor |

# 1 INTRODUCTION

## 1.1 EPIDEMIOLOGY OF CHRONIC KIDNEY DISEASE

Globally over 850 million people, with a prevalence of about 10.4% in men and 11.8% in women, are estimated to have a chronic kidney disease (CKD) (1-3). Diabetes mellitus, glomerulonephritis, hereditary kidney disease, and hypertension are the most common underlying causes. The prevalence of CKD varies between parts of the world, but the global burden of CKD is continuing to increase mainly due to the increasing prevalence of diabetic kidney disease and obesity (4, 5). Most people with CKD are over 65 years of age (6, 7). Despite a higher prevalence in women, it is men and individuals under 65 years who are more likely to progress to end-stage renal disease (ESRD) (8).

Cardiovascular disease (CVD) is a major comorbid condition in CKD and accounts for the increasing mortality globally in the CKD population in addition to infections and malignancies (9, 10). CKD is currently the 10$^{th}$ cause of death in high-income countries and is projected to become 5$^{th}$ leading cause of death worldwide in 2040 (1, 2, 11). In Sweden, nearly 600 000 people have CKD.

Due to the lack of effective therapies, many patients develop ESRD, a condition treatable only with dialysis or kidney transplantation which are expensive treatment options. Globally 2.5 million require dialysis for their survival and 1.4 million people live with a kidney transplant (3). In Sweden there are close to 4000 dialysis patients and over 5000 transplanted individuals (12). The yearly mortality in dialysis in most developed countries is still around 20%, which is similar to many metastatic cancers (13). In dialysis patients over 70 years survival is similar to that of pancreas and lung cancer (14). Comorbidities complicating treatment and reduced quality of life are not unusual (5).

## 1.2 EPIDEMIOLOGY OF HYPERTENSION

According to the World Health Organization (WHO) about 25 % of the adult population in the world suffer from hypertension (15), which would translate to more than 2 million people in Sweden (16). By the age of 65 years, 50 % of the population in Sweden are hypertensive and close to half of those are believed to be undiagnosed (17). The etiology of approximately 90% of hypertension is not well defined and is therefore classified as essential hypertension (EHT). Hypertension is the most important risk factor for cardiovascular diseases, primarily stroke but also heart failure, coronary artery disease, and kidney disease. Risk factors for developing EHT are unhealthy diets, physical inactivity, being overweight or obese, or tobacco and alcohol use. Non-modifiable risk factors are age and genetic factors (18).

The prevalence of obesity and overweight in the world has tripled since 1975. Close to 40% of adults over 18 year are overweight and 13% are obese. Globally there are more people who are obese and overweight than underweight. Furthermore, obesity and overweight are linked to more deaths worldwide than underweight (19). The long-term consequences of

cardiovascular disease (CVD), type 2 Diabetes Mellitus (T2DM), and certain forms of cancer increase morbidity and mortality rate among obese individuals (20, 21).

Secondary hypertension is defined as elevated blood pressure with co-existing diseases such as kidney disease or diabetes mellitus, and accounts for approximately 10% of all hypertension. Secondary causes should also be considered in resistant hypertension, early onset, or malignant hypertension events as well as primary aldosteronism, pheochromocytoma, hyperthyroidism, renal artery stenosis, and renal vascular disease. In recent years obstructive sleep apnea (OSA) has been identified as a common cause of hypertension (22).

# 2 BACKGROUND

## 2.1 INFLAMMATION

### 2.1.1 Acute inflammation

The word inflammation is from Latin (inflammatio) and means firing or burning. The classical signs of acute inflammation are redness (rubor), swelling (tumor), pain (dolor), local heat increase (calor), and impaired function (functio laesa). The inflammatory response consists of both cellular and humoral components and actions. The acute inflammatory response provides protection following tissue injury and infection by restricting damage to the local site, recruiting immune cells to eliminate the invading pathogen, and initiating the process of wound repair. (23, 24).

The acute inflammatory reaction is initiated by tissue damage caused by e.g. trauma, heat or cold exposure, ischemia-reperfusion, invasion of bacteria, viruses, or other microorganisms. Exposure of cell debris in the tissue activates pattern recognition receptors (PRR), for example toll like receptors (TLR), on macrophages, that initiate several cascade reactions. Local production of bradykinin promotes vasodilatation and permeability that is further enhanced by complement factors (C3a and C5a) binding to mast cells that release histamine, prostaglandin, and leukotrienes. The two latter also stimulate chemotaxis for neutrophils. Endothelial cells produce adhesion molecules that binds to circulating neutrophils which then migrate to the damaged area. Neutrophils start phagocytosis and release of inflammatory mediators that attracts macrophages into the tissue and circulating monocytes. Macrophages produce inflammatory cytokines such as interleukin (IL)-1, IL-6, and TNF (25, 26). These cytokines stimulate coagulation and increase vascular permeability. IL-1 and TNF also induce increased expression of adhesion molecules on endothelial cells, allowing for circulating neutrophils, monocytes, granulocytes, and lymphocytes to interact with the endothelium and extravasate into the inflamed tissues. Monocytes are differentiated into macrophages in the tissue. Macrophages also produce other proinflammatory mediators e.g. growth factors, that affect fibroblasts to produce collagen and stimulate other macrophages

and lymphocytes to maintain the release of mediators and thereby inflammation. The collagen production by fibroblast results in scar healing (23, 27).

Acute respiratory distress syndrome (ARDS) and sepsis are two conditions in where there is an inadequate inflammatory response. In ARDS the inflammation is sustained by immune cells in the lung tissue and in sepsis proinflammatory cytokines are produced and excreted in an excessive amount causing a "cytokine storm" (28, 29)

### 2.1.2 Chronic inflammation

Chronic inflammation is not primarily defined by the duration of the inflammatory condition but also by the immune cells present in the tissue such as lymphocytes, macrophages, and plasma cells that mature into B-cells. An inflammatory response includes actions of both innate immune cells and immune cells that contribute to the acquired or adaptive immune response. Thus, the immune cells that maintain chronic inflammation are activated as a part of the acute inflammatory response although with some delay. If the inflammatory stimulus is not controlled, the inflammation can become chronic and is consequently sustained by the immune system (Fig 1) (30, 31).

Inflammation has during recent decades been increasingly recognized as an important causal or contributing factor in many acute and chronic diseases e.g. myocardial infarction, T2DM, hypertension, heart failure, CKD, and RA (18, 30-32).

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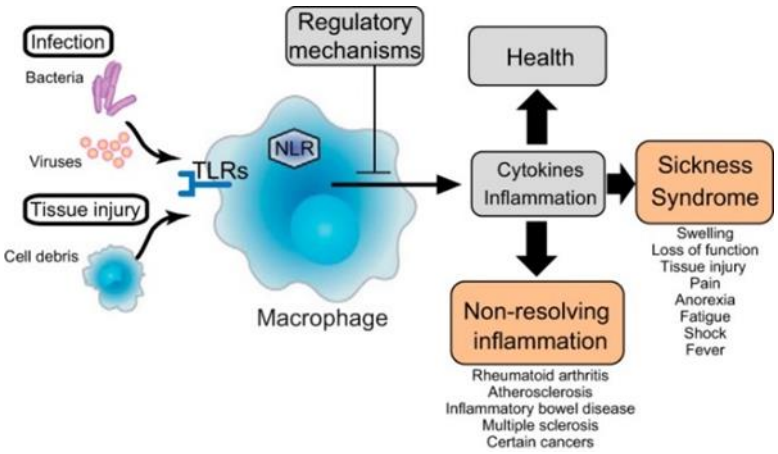



**Fig 1**. *A schematic illustration of the possible course of inflammation. TLR – Toll like receptor, NLR -nucleotide-binding oligomerization domain receptor. TLR and NLR are examples of PRR. Sundman (33). Reprinted with permission from The American Physiological Society*

## 2.2 THE AUTONOMIC NERVOUS SYSTEM

### 2.2.1 Anatomy and function

The purpose of the autonomic nervous system (ANS) is to secure and maintain a stable physiological balance - homeostasis - while the body is constantly affected by external and internal factors. The hypothalamus, the limbic system, the medulla etc. are constantly processing information from the internal and external milieu. They distribute nerve signals that regulate organ and tissue function whose activity is essential but not guided by

consciousness. Regulatory signals are also largely influenced by neural reflexes where supraspinal involvement is not required (34).

ANS is divided into the sympathetic nervous system (SNS) and the parasympathetic nervous system (PNS). The two systems supply all organs and tissues in the thoracic and abdominal cavities; the heart, the lungs, the liver, the kidneys, the spleen, the intestines etc. Exceptions from dual innervation are the adrenal marrow, the sweat glands, the blood vessels of the skin, and smooth muscles which have only sympathetic innervation (Fig. 2). Sympathetic and parasympathetic nerves usually have the opposite effects on target organs and classically, the sympathetic nervous system is usually associated with "fight and flight" and the parasympathetic nervous system with "rest and digest" (35).

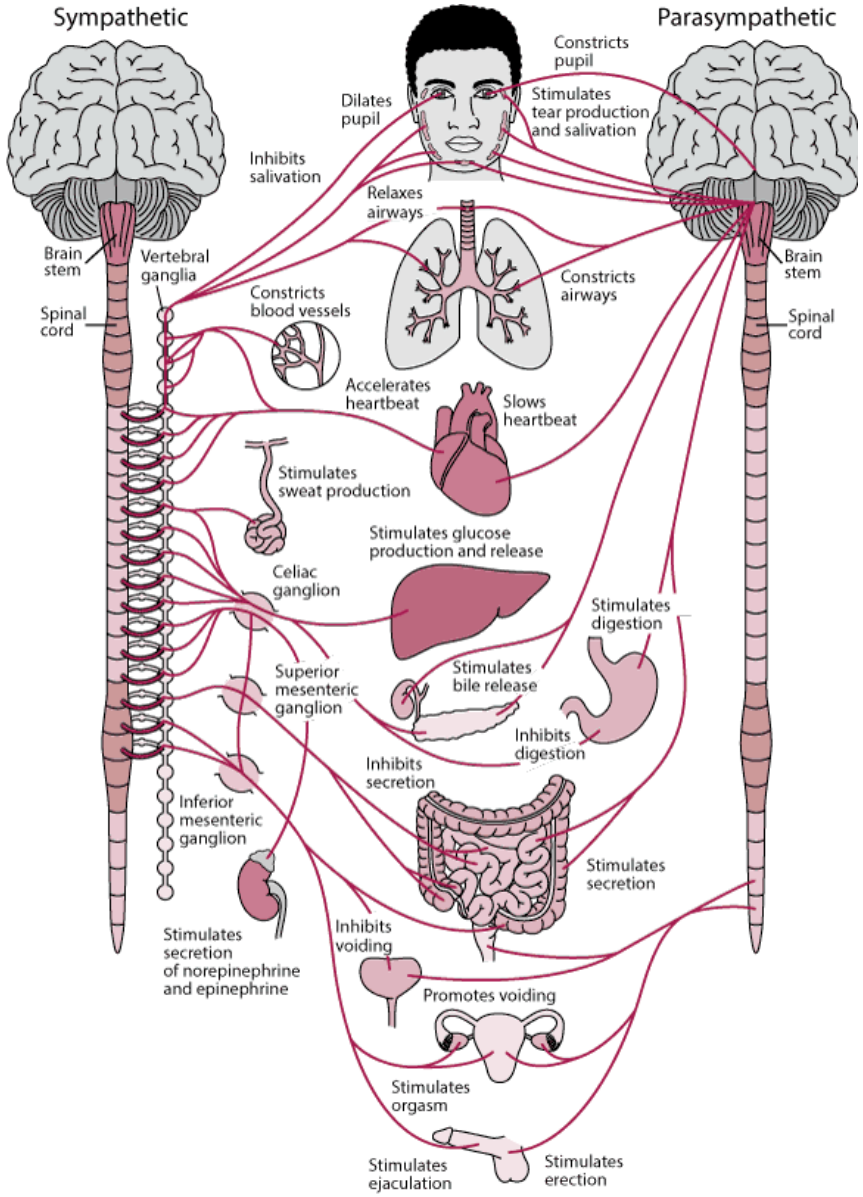



**Fig 2.** *The autonomic nervous system illustrated with the sympathetic and the parasympathetic divisions and schematic overview of innervation by the two systems. The sympathetic cell bodies are found in the thoracic and upper lumbar part of the spinal cord. The parasympathetic nerves originate from cranial nerves and lower lumbar part of the spinal cord. Image from MSD Manual. Reprinted with permission from Merck & Co., Inc., Kenilworth, NJ*

#### *2.2.1.1 Efferent motor nerves*

The ANS cell bodies are in the cranial nerves and spinal cord. The nerve threads, called axons, connect to ganglia and create synapses. The postganglionic axon then extends to an effector site. In the SNS, the ganglia are close to the spine, while the parasympathetic ganglia are located closer to the target organ. The transmitter substance in all preganglionic nerves and parasympathetic postganglionic nerves is acetylcholine that, in the synapses of the ganglia, binds to a nicotinic receptor and in the postganglionic synapses to a muscarinic receptor. In sympathetic postganglionic synapses, adrenaline or norepinephrine (NE) is released and bind to receptors in the target organ (36).

The sympathetic efferent, outgoing motor nerve cell bodies are in the thoracic and upper lumbar parts of the spinal cord. The preganglionic axons form a chain of ganglia that runs along the spine on both sides, called the sympathetic trunk. The postganglionic axons are distributed to the face, heart, lungs, kidneys etc. and mediate a regulating effect (37).

The parasympathetic preganglionic nerve cell bodies are in the cranial nerves III (Oculomotor nerve), VII (Facial nerve), IX (Glossopharyngeal nerve), X (Vagus nerve), and nerve nuclei in the medulla oblongata and in the sacral part of the spinal cord. The parasympathetic ganglia are located near or in the target organ (36, 38).

#### *2.2.1.2 Afferent sensory nerves*

Afferent, incoming sensory axons, have cell bodies in sensory ganglia in the spinal cord and cranial nerves. Visceral afferent parasympathetic axons in the glossopharyngeal nerve and vagal nerve convey information from the heart, vessels, lungs, and gastrointestinal tract to the brainstem via the inferior glossopharyngeal ganglion and inferior vagal ganglion (nodose ganglion). The afferent sensory nerves are also included in reflexes that control blood pressure, respiration, and heart rhythm via pressure-sensitive baroreceptors and hypoxia-sensitive chemoreceptors in the aorta and carotid artery (34, 36, 38-40).

### 2.2.2 Autonomic dysfunction

Autonomic dysfunction is characterized by an imbalance between sympathetic and parasympathetic nerve activity. It is common in conditions such as CVD, congestive heart failure, and septicemia and has been associated with adverse outcome (41-43). Cardiovascular autonomic dysfunction has been demonstrated not only in diabetes but also in early stages of glucose intolerance and metabolic syndrome (44).

Autoimmune diseases characterized by chronic inflammation such as rheumatoid arthritis (RA) and systemic lupus erythematosus are associated with autonomic dysfunction and a reduction in vagus nerve activity (45, 46). Autonomic dysfunction is also a common finding in patients with CKD and is in hemodialysis patients associated with poor prognosis (47-51).

## 2.3 HEART RATE VARIABILITY

### 2.3.1 Definitions

Heart rate variability (HRV) analysis based on ECG recordings is one of the most common method to assess the ANS regulatory activity (52). Traditionally SNS and PNS have been described as two opposing structures where SNS provides stress reactions and PNS relaxation (52). Other factors that have an impact on HRV are metabolic (diabetes), endocrinal (cortisol), and barometric (orthostatic) (53). HRV is significantly influenced by sex, age, physical fitness, clinical comorbidities, smoking, and medication (54-57). Alterations in HRV can also be noted with changes in resting state, body position, and breathing pattern which may reduce the specificity of HRV for monitoring physiological or pathological situations (53, 58). A non-invasive ECG for HRV analysis is typically recorded for 1 minute up to 24 hours or longer and quantifies the amount of the interval changes between heartbeat cycles. A common approach is to use shorter recordings, e.g. 20 min divided into 5 minutes epochs. This procedure delivers reliable HRV data except for circadian rhythm that requires at least 24-hour recordings (53).

The intrinsic heart rate from the sinus node is approximately 95 to 110 beats per minute (BPM). However, the parasympathetic (vagal) efferent influence is greater than the sympathetic, which results in approximately 60-80 BPM for an adult in a resting supine position. There are several ways to quantify HRV which reflect differences in the interval between heart beats. Three different categories are used; time domain, frequency domain, and non-linear variables (Tab.1).

| | HRV indices | Unit | Definition |
|---|---|---|---|
| Time domain | NN | | Normal RR-intervals |
| | SDNN | ms | Standard deviation of all normal intervals |
| | SDANN | ms | Standard deviation of the average NN intervals in 5 minutes segments ECG |
| | RMSSD | ms | Root mean square successive difference, the square root of the mean of the squared differences between adjacent NN. |
| | pNN50 | % | Percent of differences between adjacent NN greater than 50 ms |
| | NN50 | ms | Number of adjacent NN greater than 50 ms. |
| Frequency domain | Total power | $ms^2$ | The power (variance) in the heart period power spectrum up to 0.4 Hz. |
| | Ultra low Frequency (ULF) power | $ms^2$ | The power (variance) in the heart period power spectrum up to 0.0033 Hz. |
| | Very low frequency (VLF) power | $ms^2$ | The power (variance) in the heart period power spectrum from 0.0033 to 0.04 Hz. |
| | Low Frequency (LF) power | $ms^2$ | The power (variance) in the heart period power spectrum from 0.04 to 0.15 Hz. |
| | High Frequency (HF) power | $ms^2$ | The power (variance) in the heart period power spectrum up from 0.15 to 0.4 Hz. |
| | LF/HF | | Ratio low frequency to high frequency. |

**Table 1**. *HRV. Time and frequency domain variables, units and definitions.*

Standards for measurement, interpretation, and normal values for HRV time- and frequency domain in short-term (5 min) and long-term (24 hrs.) ECG recordings, were established in 1996 by the Task Force set up by the European Society of Cardiology and the North American Society of Pacing and Electrophysiology (59). In this thesis, time and frequency domain variables will be used plus "non-linear" variables SD1 and SD2 derived from a Poincaré plot. SD 1 is an index of short-term HRV and equals RMSSD and SD2 is an index of long-term HRV and equals SDNN (60).

### 2.3.2 Heart rate variability and inflammation

The heart rate and cardiac output are influenced by the motor vagus nerve. Therefore, HRV, which is known as a reliable indicator of cardiac vagal activity, might also be related to degree of inflammation. An association between HRV and pro-inflammatory markers such as C-reactive protein (CRP), TNF, and interleukin (IL)-6 has been reported in several studies (61, 62). This is consistent with the role of the vagus nerve in the cholinergic anti-inflammatory pathway (CAP) (63). Reduced HRV is associated with adverse outcomes in hypertension, systemic inflammation, depression, CKD, and is also associated with an increased risk of sudden death (41, 64-67).

## 2.4 THE KIDNEYS

### 2.4.1 Anatomy and physiology

The main function of the kidneys is to produce urine and thereby regulate fluid balance by tightly controlling electrolytes and removing waste products from the circulation. In each kidney there is about 1 million nephrons, which is the functional unit, where blood from the renal arterial circulation is filtered across the capillary walls (Fig. 3). The filtrate, that consists of approximately 180 Liters per day, is subsequently transported and reabsorbed in the tubules and collecting ducts. The final product urine is typically between 1.5-2 Liters per day (68).

The kidneys also regulate blood pressure by several mechanisms which includes the Renin Angiotensin Aldosterone System (RAAS) and ANS activity. In the juxtaglomerular apparatus, in the glomerulus, there are specialized cells that register sodium concentration in the tubules and the volume of blood flow in the efferent arteriole. Blood volume depletion induces secretion of renin that results in vasoconstriction in the afferent arteriole and increases in sodium reabsorption via both Angiotensin II and Aldosterone systems. (68).

The kidneys secrete the hormone erythropoietin (EPO) that stimulates the bone marrow to produce red blood cells. Furthermore, the kidneys are essential for activation of vitamin D from its 25-hydroxyvitamin form to 1,25-dihydroxycholecalciferol, which is biologically active and crucial for calcium homeostasis and bone metabolism (69, 70).

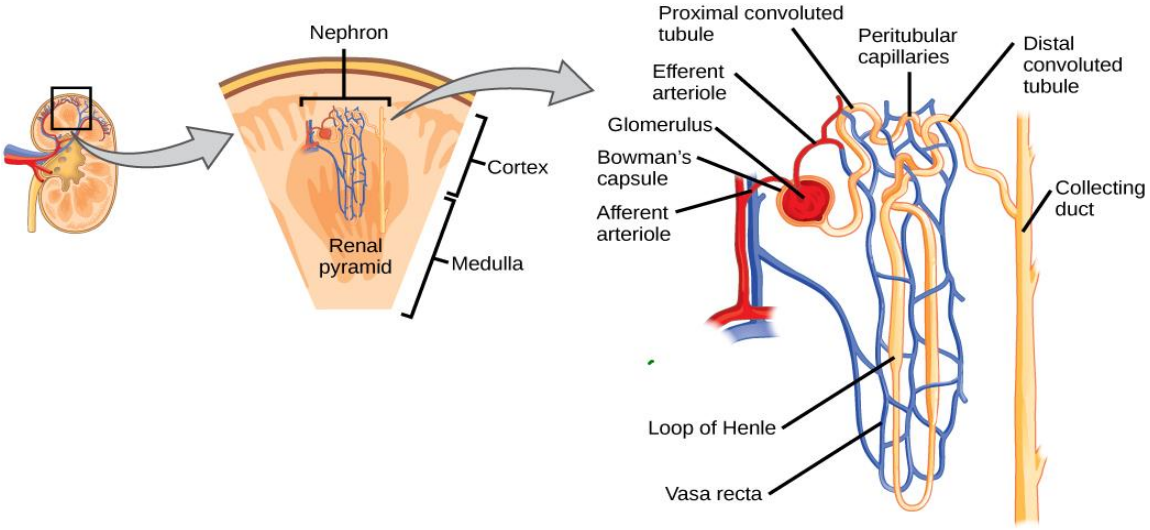



**Fig. 3** *The kidney with the nephron and enlarged nephron with anatomical structures. Image by CNX OpenStax, Wikimedia.org*

### 2.4.2 Innervation of the kidneys

Efferent sympathetic nerves are widely distributed in the kidneys with the highest concentration in the arterioles but are also present in the tubular epithelium. Increased sympathetic activity stimulates renin release, decreases blood flow, and influences the tubular epithelium to reduce sodium excretion (71). Sensory afferent sympathetic nerves are mainly concentrated to the pelvis as free nerve endings and are sensitive for pressure, i.e. urine volume (72, 73). The renal artery is mainly innervated by sympathetic efferent nerves and approximately 80% of them are found in the proximal and middle part, although efferent nerve endings in the distal part are located closer to the arterial lumen. Afferent sensory nerves are considerably fewer but equally distributed in all parts of the renal artery (74).

### 2.4.3 Kidney disease - diagnosis and definition

A routine blood test of creatinine in serum (S-) or plasma (P-) is a standard analysis in many clinical situations. Increased levels are often the first sign of kidney disease and kidney dysfunction. Creatinine, however, is affected by factors such as muscle mass, age, and hydration status which is why measuring or estimating glomerular filtration rate (GFR) is more reliable. The "golden standard" to measure GFR is inulin-clearance and iohexol clearance but they are both rather cumbersome methods. Estimation formulas based on creatinine and/or cystatin C have been developed for clinical use (75-77).

Kidney disease as well as CKD are divided in five GFR groups according to criteria from Kidney Disease: Improving Global Outcomes (KDIGO):

- normal GFR but with signs of kidney disease such as urinary abnormalities for instance proteinuria/ CKD 1 (GFR>90 ml/min/1.73m$^2$ )
- mildly decreased GFR/ CKD 2 (GFR 60-89 ml/min/1.73 m$^2$)
- moderately decreased GFR/ CKD 3 (GFR 30-59 ml/min/1.73 m$^2$)

- severely decreased GFR/CKD 4 (GFR 15-29 ml/min/1.73 $m^2$)
- kidney failure7CKD 5 (GFR < 15 ml/min/1.73 $m^2$ ) (75).

Kidney disease is also typically divided in acute kidney injury (AKI), acute kidney disease (AKD) and chronic kidney disease (CKD)

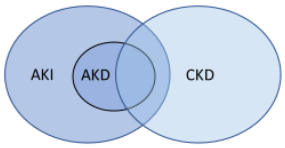



**Fig 4.** *Kidney disease divided in AKI, AKD and CKD and the overlap of conditions*

### 2.4.4 Acute kidney injury

Acute kidney injury (AKI) is a very common event in hospitalized patients and mortality is increased irrespective of the underlying cause (78-81). Definition of AKI according to KDIGO is;

- Increase of creatinine ≥ 26,5mol/L within 48 hours or
- Increase of creatinine > 1.5 x baseline assumed or known to have occurred within the prior 7 days or
- Urine production < 0,5 ml/min/kg/h for six hours (75)

Risk factors for AKI are dehydration/hypovolemia, high age, heart failure, CKD, diabetes, and anemia (81-84). Triggers of AKI are often divided in; pre-renal causes such as sepsis, shock, malignant hypertension, intoxication, and surgery; post-renal causes such as hydronephrosis, malignancies, and infections, and renal causes for instance vasculitis, rapidly progressive glomerulonephritis, and toxicity to the kidney e.g. nephrotoxic drugs (80, 81). AKI often leads to acute tubular necrosis that is a clinical event when the tubular epithelium is damaged and loses its function. AKI can heal and kidney function be restored. However, depending on the cause and magnitude of the injury and/or preexisting renal failure, there may be a permanent decrease in kidney function (85) (Fig. 5).

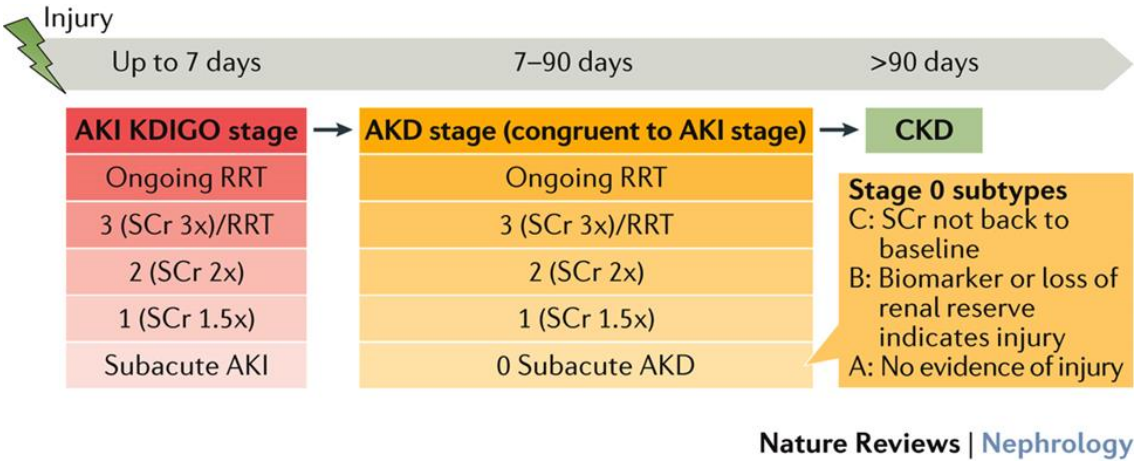



**Fig. 5** *Acute kidney disease and renal recovery. (RRT-renal replacement therapy, SCr-serum creatinine) consensus report of the Acute Disease Quality Initiative (ADQI) 16 Workgroup. Y Chawla et al. (85). . Open access*

### 2.4.5 Chronic kidney disease

CKD implies persistent changes in morphology and function of the kidneys for more than 3 months (KDIGO). CKD is, as previously described, divided into 5 stages that is based on GFR.

Advanced CKD often involves a number of disturbances such as fluid volume overload, electrolyte imbalances, anemia, malnutrition, CKD–mineral and bone disorder but also includes cognitive dysfunction and chronic inflammation (86, 87). Untreated chronic inflammation per se, contributes to cardiovascular morbidity and mortality in dialysis patients (87, 88). Pre-dialysis fluid overload is also associated with inflammation (89).

Progression to CKD 5 and end-stage renal disease (ESRD) is followed by initiation of renal replacement therapy (RRT). Either hemodialysis (HD), peritoneal dialysis (PD), or kidney transplantation. The Swedish Renal Registry (SRR) coverage rate for RRT patients is nearly 100 percent and reports annually epidemiology, prevalence, incidence, and several quality parameters. Figure 6 shows the underlying diseases for CKD among all RRT patients (9918 persons) included in the registry in December 2017 (12).

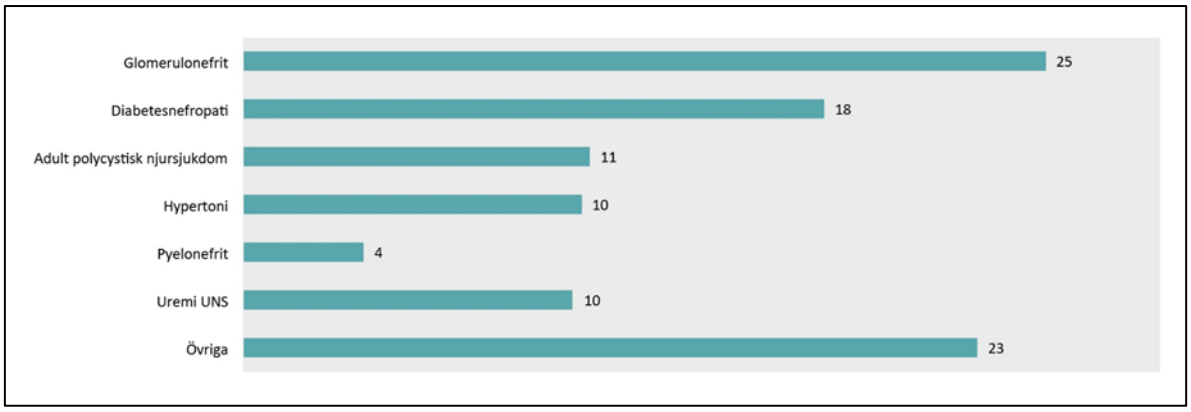



**Fig. 6** *Prevalent patients in RRT by diagnosis (%). Glomerulonephritis 25%, diabetes nephropathy 18%, Aduöt polycystic kidney disease 11%, Hypertension 10%, Pyelonephritis 4 %, Uremia without specification 10% and others 23 %. SRR Annual report 2018. Reprinted with permission from MedSciNet AB.*

### 2.4.6 Chronic kidney disease and inflammation

The prognosis and outcome for CKD and ESRD-patients is partly dependent on the underlying disease but, there is a substantial impact of inflammation both in CKD progression, co-morbidity, and mortality. Inflammation in CKD was described to be associated with CVD and mortality at the end of the last century. Since then it has become clear that inflammation is an entity per se in CKD (90, 91). The causes behind increased inflammatory activity in CKD patients are numerous, e.g. underlying diseases, lifestyle factors, reduced elimination of cytokines due to renal failure, metabolic acidosis, and more (92). The uremic environment induces oxidative and carbonyl stress and substantially promotes inflammation (93, 94). Reduced levels of both 25-hydroxy vitamin D and 1,25-dihydroxycholecalciferol similarly affects the functionality of both the innate and adaptive

immune system (95). Studies have shown that IL-6 is the best predictor of all-cause mortality and CVD mortality even though CRP, IL-1, TNF, and other have an impact and similarly an negative correlation to GFR (96-98).

Malnutrition and protein wasting in CKD are explained by several factors e.g. depression and anorexia because of circulating cytokines in the brain, and suppressed levels of anabolic hormones. Malnutrition and protein wasting are also shown to be driven by persistent inflammation (99, 100). Moreover, inflammation maintain anemia in CKD-patients which is further amplified by decreased synthesis of EPO and lower responsiveness to EPO in the bone marrow (101).

## 2.5 HYPERTENSION

### 2.5.1 Definition and pathophysiology

According to 2018 European Society of Cardiology (ESC) and European Society of Hypertension (ESH) guidelines, hypertension is defined as an office systolic blood pressure (SBP) of $\geq 140$ mmHg and/or diastolic blood pressure (DBP) of $\geq 90$ mmHg, independent of age, sex, and comorbidities (102). BP should be measured with auscultatory or oscillometric semiautomatic or automatic sphygmomanometers at two separate visits (103).

The historical explanation of the cause of both hypertension and the origin of hypertension related organ damage has mainly been focused on a viscous circle of sustained sympathetic activation and the mechanical wear of the blood vessels causing arterial stiffness and cardiac damage (104). Heart rate, HRV, and blood levels of norepinephrine (NE) have traditionally been used to quantify the adrenergic activity in hypertension (64). In addition to the ANS other "intrinsic" regulators such as the RAAS and the Endothelin-systems have crucial role in blood pressure pathophysiology.

Studies have shown that muscular sympathetic nerve activity (MSNA), measured as burst per minute with microneurography, gives further information about the development of EHT. A meta-analysis showed that EHT patients had higher MSNA both when untreated and treated with antihypertensive drugs, suggesting that they did not fully obtain a normal circulatory sympathetic drive despite therapy (105). In other studies the MSNA was inversely related to HR which may indicate an greater parasympathetic (vagal) impact of HR than previously assumed (106).

### 2.5.2 Hypertension and placental growth factor

Placental growth factor (PlGF) is an angiogenic growth factor of the Vascular Endothelial Growth Factor (VEGF) family. It has pro-angiogenic effects on the fetoplacental circulation and supports trophoblast growth. Circulating PlGF levels depend on gestational age during pregnancy and particularly lower levels are associated to preeclampsia. It has been demonstrated that PlGF interacts with a neuro-immunological pathway in the spleen which suggests a potential immunological trigger of hypertension (107-109).

A novel mechanism relying on the neurosplenic sympathetic drive using a deoxycorticosterone acetate (DOCA)-salt hypertensive animal model has been shown to contribute to increased BP. DOCA-salt challenge in this model, significantly amplified PlGF expression in the spleen, which adds further clues to a neuro-splenic immune system involvement (110).

### 2.5.3 Hypertension and renal denervation

According to ESC/ESH 2018 guidelines resistant hypertension is to be considered when a treatment strategy consisting of appropriate modification of lifestyle measures and treatment with optimal or best-tolerated doses of three or more drugs, which should include a diuretic, is ineffective in lowering office SBP and DBP values to < 140 mmHg and/or < 90 mmHg, respectively (102).

Modulation of renal sympathetic nerve activity through catheter-based radiofrequency ablation in resistant hypertension in humans was first described in 2009 (111). In the European Symplicity-2 study this invasive add-on treatment was evaluated. Primary endpoint was office BP reduction (≥ 10 mmHg) at 6-months after denervation and was achieved in 80-90 % of patients without altering renal function (112). However, the Symplicity-3 study, conducted in the United States, that compared renal denervation with a sham procedure, failed to demonstrate a significant BP effect (113). Hence the use of RDN ended in most countries but the research did not. Two randomized trials aiming at renal denervation in hypertensive patients off any medication (SPYRAL HTN-OFF MED) or on (SPYRAL HTN-ONMED) demonstrated a significantly BP decrease at 3 months using a multielectrode catheter (114, 115). In 2018 the RADIANCE-HT-SOLO showed that endovascular ultrasound RDN reduced BP at 6 months requiring fewer antihypertensive medications compared with a sham control.

ESC/ESH 2018 guidelines do not currently recommend invasive device-based treatment but are awaiting ongoing trial results for future guidance.

### 2.5.4 Hypertension and inflammation

Over the last decades it has become more evident that the immune system plays an important role in both pathogenesis and maintenance of EHT. There are several experiments that illustrate the role of different immune cells in hypertension. Models of salt-depending hypertension or angiotensin II (ang II) induced hypertension have shown that dysfunctional monocytes/macrophages are protective (116-118). Furthermore, thymectomy in hypertensive models reduces BP (119, 120) Patients with human immunodeficiency virus and no anti-viral medication are less likely to develop hypertension as compared to HIV-negative individuals, or HIV-positive subjects with highly active antiretroviral therapy (HAART) (121). Another study demonstrated that hypertension was not developed in a model with a deficient gene transcoding enzyme resulting in lack of B and T-cells, when exposed for Ang II (122). T-cells have been shown to moderate endothelin dysfunction and microvascular injury caused by

Ang II. Lack of regulatory T-cells have been shown to exaggerate Ang II-induced microvascular injury by modulating innate and adaptive immune responses (123)

In figure 7 the role of immune cells that can trigger and maintain hypertension is illustrated. Environmental factors and genetic susceptibility lead to a small BP augmentation by activating the SNS and inhibiting the PNS. High BP and/or pro-hypertensive stimuli induce tissue injury that leads to development of Damage-associated molecule patterns (DAMPs). DAMPs activate innate immunity via toll-like receptors (TLR) on type 1 macrophages (M1), type 1 dendritic cells (DC1) and natural killer (NK) cells. Innate immune cells contribute to inflammation, both directly and via adaptive immunity and the production of autoantibodies, leading to vascular injury that closes the pro-hypertensive loop which is a feed-forward process resulting in progressive BP elevation. Throughout this process, anti-inflammatory cells could provide homeostatic fine-tuning of the inflammatory process in blood vessels and the kidney. However, this anti-inflammatory mechanism could be rendered incapable of counteracting the development of hypertension because of factors such as Ang II. PAMPs are Pathogen-associated molecular patterns derived from bacteria, viruses, and other micro-organisms (124, 125).

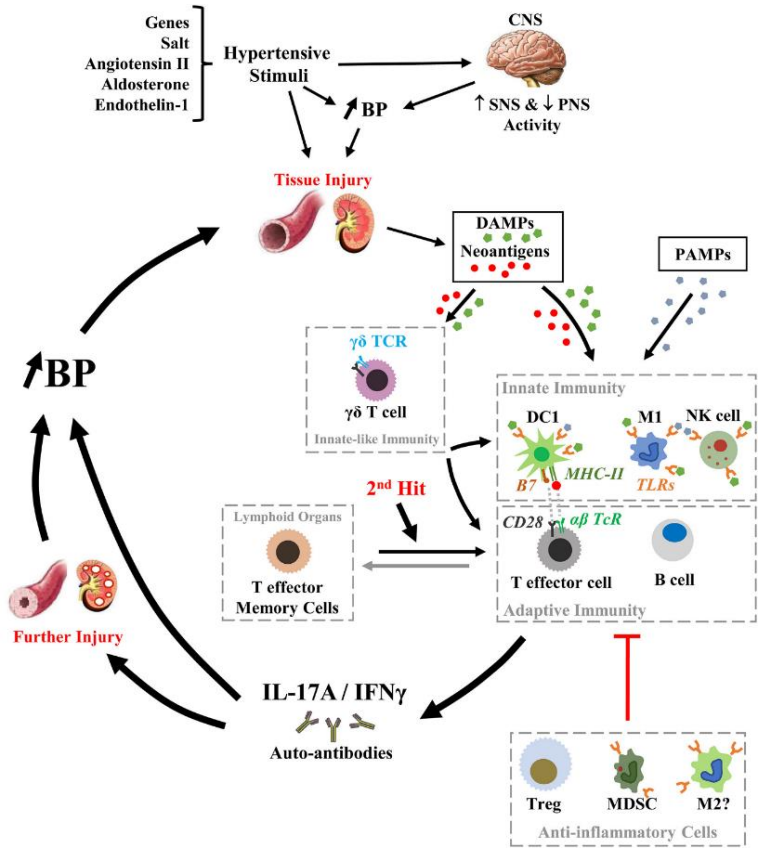



**Fig 7**. *The viscous circle of hypertension and inflammation. Caillon (124). Reprinted with permission from Whiley and Sons*

## 2.6 THE CHOLINERGIC ANTI-INFLAMMATORY PATHWAY

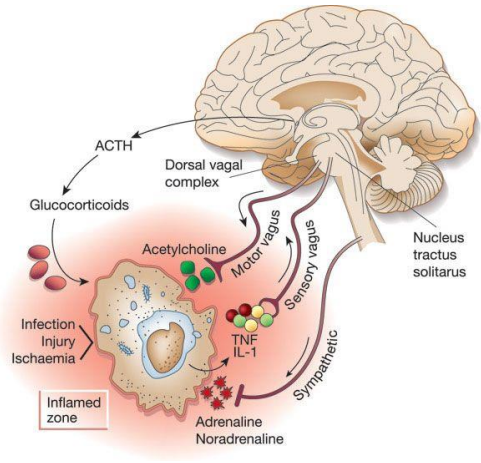
### 2.6.1 The vagus nerve

Vagus means wandering and the parasympathetic vagal nerve is the longest and most widespread of all nerves in the body. The vagus nerve is the 10$^{th}$ cranial nerve and is a mixed nerve meaning that it carries both sensory afferent (80%) and efferent motor fibers (126). Vagus is vital as it provides parasympathetic innervation to all thoracic organs and almost all subdiaphragmatic, visceral organs. The axons to the thoracic organs are coated with myelin which makes the signal transport fast whereas the axons to visceral organs are unmyelinated and hence slower (38, 127).

The afferent axons send sensory signals from the visceral organs and terminate in the Tractus Solitarius in the brainstem where signals are subsequently forwarded to other structures in the brainstem, thalamus and the forehead areas. The efferent motor vagus neurons begin in the dorsal motor nucleus (DMN) and the nucleus ambiguus (NA) in the medulla oblongata. The parasympathetic axons meet and interact with postganglionic neurons in the ganglia in the proximity of or in the target organ (34, 36).

### 2.6.2 Description of the cholinergic anti-Inflammatory pathway

In a highly cited paper in Nature 2002, K Tracey and co-workers, described the CAP and the anti-inflammatory reflex for the first time. In case of injury, inflammation, ischemia, or infection signals are relayed to the central nervous system via the sensory afferent vagus nerve. An activating response is subsequently initiated and returned via the efferent motor part of the vagus nerve, referred to as the inflammatory reflex (Fig. 8) (26). In a proof-of-concept study is was demonstrated that VNS attenuates the systemic inflammatory response to endotoxin (128, 129). Since these seminal papers were published continued research in the field has delineated many of the detailed meticulous mechanisms and substances involved in this neuroimmune pathway. **Fig 8** *CAP 2002 Tracey (26). Reprinted with permission from Springer Nature*




The efferent motor vagus terminates in the sympathetic celiac plexus where the splenic nerve is activated. (130, 131). Specialized T-cells, with the ability to produce the enzyme acetylcholine transferase (ChAT-cells), are located in the red pulp and marginal zone of the spleen (132). ChAT-cells are stimulated by beta-adrenergic receptors that binds NE originating from the splenic nerve. Activated ChAT-cells synthesize and release acetylcholine that binds to an α7nAChR on macrophages and thereby downregulates TNF release. Released NE also inhibits B-cell migration and antibody production (132-135) (Figure 9).

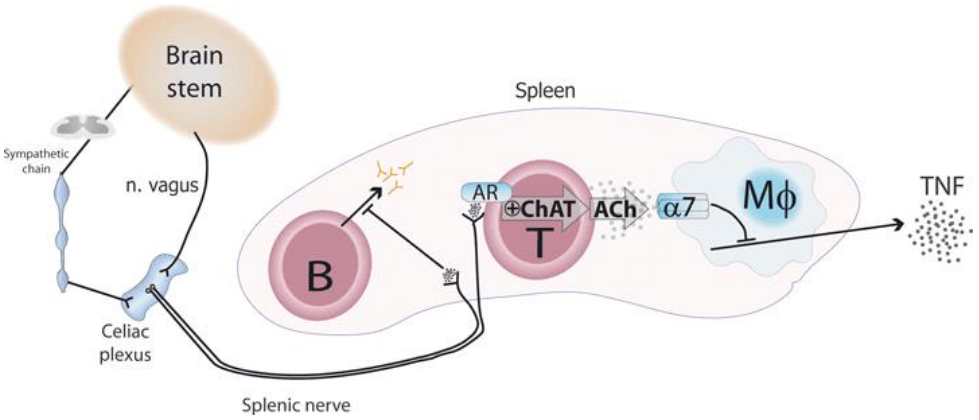



**Fig 9**. *Hosoi (133) Reprinted with permission from John Wiley and Sons*

In addition to receptors for inflammatory cytokines, afferent nerve endings have PRR, such as TLRs for PAMPs and DAMPs and receptors for serotonin, histamine, prostaglandin, and nerve growth factor (136, 137). There are also nociceptive receptors, but their signal reaches primarily the CNS (138, 139). Adrenergic and acetylcholine receptors are expressed by immune cells and facilitates neuroimmune communication (140-142). Immune cells can, in addition to neuro transmitter substances as acetylcholine, also synthesize dopamine and catecholamines and thereby participate in the local regulation of neuroimmune signaling (135, 141).

The role of α7nAChR has furthermore been examined in several studies, for example in an α7nAChR depleted mice model. When inducing arthritis in this model by immunization of collagen II, the α7nAChR depleted mice showed milder arthritis and cartilage destruction as well as a decrease in T-cells in lymph nodes. This result indicates that the α7nAChR has an important role in the regulation of inflammatory response as well as in T-cell dependent reactions both in the innate and adaptive immune system response (32).

### 2.6.3 Vagus nerve stimulation

Neuromodulation by vagus nerve stimulation (VNS) was first recorded in the late 1800's when it was observed that mechanical stimulation of the vagal region suppressed seizure activity (J. Corning, Prolonged instrumental compression of the primitive carotid artery as a therapeutic agent., Med. Rec. 21, 173–174 (1882)).

VNS by physiological and pharmacological modalities has been studied in several animal models and has been shown to attenuate inflammatory markers such as High mobility group Box-1 protein (HMGB1) and TNF in models of inflammation, including collagen induced arthritis (128, 129, 140, 143).

Cholinergic analogues, such as GTS 21, have been used to explore how CAP performs in different experiments simulating clinical situations. In a model of endotoxemia, administration of GTS 21 improved survival and reduced TNF in treated animals as compared with controls (143). In a pancreatitis model histological score was improved and

IL-6 in the circulation was decreased (144). Similarly, in a model of renal ischemia, GTS 21 decreased TNF levels in kidney tissue and reduced tubular necrosis (145).

### *2.6.3.1 Invasive vagus nerve stimulation*

VNS, with an implantable device, was approved by the US food and drug administration (FDA) in 2002 as a treatment for drug resistant epilepsy (146, 147). The implantable device is a battery driven stimulator with a wire electrode attached around the left cervical vagus. In 2005 the approval was expanded to include treatment of resistant depression (Fig. 10) (148). The effect in the CNS of VNS is, in brief, an alteration of signaling/transmission in the prefrontal cortex area, midbrain, and hindbrain where NE and serotonin are transmitter substances (149).

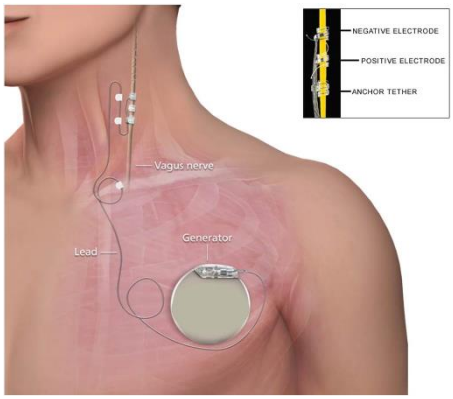



**Fig 10**. *Schematic drawing of implantable VNS. Verrier (150). Open access.*

Recent clinical studies have shown that electrical or electromagnetic energy via cervical implants stimulating the vagus nerve may be beneficial for patients with inflammatory diseases such as RA (151, 152) and Crohn's disease (153, 154). In animal models of heart failure, VNS has shown positive results for long-term survival (155), while a recent clinical study, (INOVATE-H) in 707 patients with heart failure followed for a treatment for a mean of 16 months, did not confirm any beneficial effect of VNS on survival (156). Models of myocardial ischemia-reperfusion damage have shown better outcome after VNS although results have not been consistent in other models (157).

### *2.6.3.2 Transcutaneous vagus nerve stimulation*

Electrical transcutaneous VNS (tVNS) can be directed to the outer ear, where the auricular branch of vagus has sensory fibers, or at the cervical vagus. In 2011 a tVNS device for electrical stimulation of the auricular branch of vagus was approved in Europe for treatment of drug resistant epilepsy (158). This device has also been used in clinical trials of depression and pain (159, 160) (Fig. 11a). TcVNS Gammacore, stimulates the cervical vagus and was approved in Europe in 2013 and by FDA in 2017, for the treatment of migraine and cluster headache (Fig. 11b) (161).

**Fig. 11a** *tVNS device NEMOS®. (158)*

**Fig. 11b** *tcVNS Gammacore. Practical Neurology. Reprinted wih permision from Elsevier. Open access (162).*

In a recent study by De Couck *et.al* the effect of auricular tVNS in healthy subjects was studied, focusing on HRV as an indirect measure of vagal activity. The study showed that

short term (10 min) left versus right ear tVNS versus sham resulted in significant increases only in SDNN. Prolonged tVNS, (1 hour) in the right ear, significantly increased the LF and LF/HF components of HRV and SDNN in women, but not in men (163).

In a study by Addorisio *et al.* a vibrotactile device was used to bring vibrations to the cymba choncha of the outer ear. Both healthy subjects and patients were treated and resulted in modulation of peripheral blood cytokine levels in healthy subjects and lowering of TNF levels and disease activity in patients (164).

#### *2.6.3.3 Drug mediated vagus nerve stimulation*

The idea of pharmacological stimulation of vagus is not new but quite recently a randomized, double-blind, placebo-controlled study was published. Galantamine is a centrally acting acetylcholine esterase inhibitor and typically prescribed to prevent progression of Alzheimer´s disease. Galantamine, with anti-inflammatory activity and known to active CAP, was given to patients with metabolic syndrome. Galantamine treatment for 12 weeks was shown to alleviate inflammation and alter HRV as well as lowering plasma insulin and insulin resistance (165).

# 3 AIM OF THE STUDIES

The aim of this thesis was

1. To study the functionality of the cholinergic anti-inflammatory pathway/CAP in hemodialysis patients and peritoneal dialysis patients, compared to controls, using a whole blood inflammation model.

2. To explore the hypothesis that patients undergoing RDN due to treatment-resistant hypertension would respond with reduced cytokine release through decreased sympathetic nerve activity.

3. To investigate if short-term VNS, using a minimally invasive method, could improve inflammatory cytokine levels and alter vagal tone (HRV) in hemodialysis patients.

4. To investigate correlations between inflammatory markers and HRV indices in a freely available database (HeartBEAT). To calculate the optimal length of an ECG recording that are needed when utilizing HRV methodology for predicting elevated levels of inflammatory markers.

# 4 STUDY SUBJECTS AND METHODS

## 4.1 STUDY SUBJECTS

### 4.1.1 Study I – The cholinergic anti-inflammatory pathway activity in dialysis patients: a role for immunomodulation?

Patients were recruited among chronic dialysis patients at the Renal Department at Karolinska University Hospital, Huddinge. Healthy controls were recruited among staff at the Renal Department at Karolinska University Hospital, Huddinge. Both patients and healthy controls were included by Marie Hilderman.

Inclusion criteria for patients were:

- RRT with hemodialysis (HD) or peritonealdialysis (PD) regardless of underlying disease.
- Stable dialysis procedures.
- No clinically significant symptoms of infection or inflammation.
- Ability to understand oral and written study information.

Inclusion criteria for healthy controls were:

- No significant history of inflammatory disease.
- No clinically significant symptoms of infection or inflammation.
- Ability to understand oral and written study information.

### 4.1.2 Study II – The Cholinergic anti-inflammatory pathway in resistant hypertension treated with renal denervation

All patients and disease controls were recruited at the hypertension clinic at Södersjukhuset, Stockholm by cardiologists Nils Witt, Christina Jägrén and Joakim Olbers.

Inclusion criteria for patients were:

- Office SBP > 160 mmHg despite the use of at least three anti-hypertensive drugs, including a diuretc, at maximum tolerated doses.
- No current clinical signs of infection or inflammation.
- Ability to understand oral and written study information.

Exclusion criteria for patients were:

- Renal artery stenosis.
- Secondary hypertension.

Inclusion criteria for disease controls were:

- Stable patients scheduled for elective coronary angiography due to ischemic heart disease.
- No current clinical signs of infection or inflammation.
- Ability to understand oral and written study information.

### 4.1.3 Study III – Minimally invasive vagus nerve stimulation in hemodialysis patients and inflammatory response

The study population was recruited consecutively among stable chronic hemodialysis patients at the Dialysis Department at Karolinska University Hospital, Huddinge between November 2014 and November 2015. All patients were included by Marie Hilderman.
Inclusion criteria were:

- RRT with hemodialysis regardless of underlying disease.
- Stable dialysis procedures.
- No clinically significant symptoms of infection or inflammation.
- Ability to understand oral and written study information.

### 4.1.4 Study IV – Prognostic potential of heart rate and heart rate variability for monitoring the cholinergic anti-inflammatory pathway

#### *4.1.4.1 Data source*

The database Heart Biomarker Evaluation in Apnea Treatment (HeartBEAT) has been made accessible by the National Sleep Research Resource (NSRR, sleepdata.org) USA. HeartBEAT is a randomized controlled trial that assesses the effects of supplemental nocturnal oxygen or Continuous Positive Airway Pressure (CPAP) therapy, compared to optimal medical preventive therapy in patients with CVD or CVD risk factors and moderate to severe OSA or history of snoring.
Inclusion criteria were:

- Established Coronary Artery Disease or established CVD risk factors.
- Home sleep test that showed moderately severe sleep apnea.

Exclusion criteria were:

- Poorly controlled health.
- Current use of supplemental oxygen or CPAP for OSA.

#### *4.1.4.2 Study subjects*

Three-hundred and eighteen patients aged 45-75 were included. The protocol for the trial consisted of 3 arms and subject were randomized to:
1. Healthy Lifestyle and Sleep Education (HLSE).
2. HLSE and education in CPAP treatment and usage of it for 3 months.
3. HLSE and supplemental oxygen during sleep for 3 months.

All patients received medical preventive therapy according to current American Heart Association guidelines for prevention of CVD and sleep guidelines. Patients were sampled twice; at baseline and at 3 months when 301 patients remained. The HeartBEAT database contains a large amount of widely different parameters e g socioeconomic status, medication, body composition, blood pressure, blood glucose, and lipid status. Characteristics of patients, outcomes, and results are described in detail elsewhere (166-168). For this study we used

nocturnal ECG-recordings and available inflammatory markers such as CRP, TNF, IL-6, and E-selectin. Fifty-six patients were not analyzed for HRV because of missing or noisy ECG within the target length of recording (6 ± 2 hrs).

## 4.2 METHODS

### 4.2.1 Blood sampling and ECG Study I-III

#### *4.2.1.1 Study I*

All subjects were sampled in the morning. HD-patients were sampled before dialysis. In patients treated with Continuous ambulatory peritoneal dialysis (CAPD)-patients the sampling was timed to the night bag and first day bag exchange in the morning. In patients treated with Ambulatory peritoneal dialysis (APD) samples were collected in the morning after the end of the APD session. A quiet examining room outside the Dialysis Department was used for ECG recordings, which were done at a separate visit with the subjects in a supine position in a bed.

#### *4.2.1.2 Study II*

All subjects were sampled before treatment/intervention (RDN or Coronary Angiography (CA)) in the morning at the PCI- (Percutaneous coronary intervention) unit at the Cardiology Department at Södersjukhuset, Stockholm. ECG was recorded at the same occasions with the patient in a supine position in a bed. Blood samples and ECG were again collected the day after intervention in all subjects. RDN-patient were then followed with blood samples and ECG at 3 and 6 months.

#### *4.2.1.3 Study III*

In this study all patients had blood drawn after ECG recording, but before intervention treatment at the Dialysis Department at Karolinska University Hospital Huddinge. Blood samples were collected when dialysis needles were inserted. ECG recordings were done in a quiet examining room outside the Dialysis Department with the patients in a supine position. These procedures were repeated during the treatment period after 2 and 4 weeks, and at follow up at 8 and 12 weeks (Fig. 12).

### 4.2.2 Whole blood assay Study I-III

#### *4.2.2.1 Blood samples*

Whole blood was collected into two heparinized tubes and immediately kept in a 37°C heated container until processed within 60-120 min. A non-heparinized tube was left to clot for 2 hours at room temperature (approximately 25° C) and subsequently centrifuged (5-10 min, 828 g [2000 rpm in Sorvall RT7 Plus centrifuge]). Five hundred μL aliquots of harvested serum were frozen at -80° C for pending analysis of basal cytokines.

Reagents:

- Lipopolysaccharide (LPS) from Escherichia coli 0111:B4, Sigma-Aldrich (St Louise, MO, USA), cat.No. L4130 was used. Five mg LPS was solved in 1 mL phosphate buffered saline (PBS), sonicated for 30 min, vortexed well and diluted 5 times to $1x10^6$ ng/mL (= stock solution) and then portioned and frozen at -80° C. The $1x10^6$ ng/mL stock solution of LPS was serially diluted with PBS to 10000 and 1000 ng/mL.
- GTS 21, a cholinergic analogue was provided by Yousef Al-Abed who synthesized, isolated and standardized it. Yousef Al-Abed is a medicinal chemist and investigator at the Feinstein Institute for Medical Research. GTS 21 (2 mg) was diluted in distilled water to approximately 4.6 and 9.2 mmol/L working solutions, respectively.

#### *4.2.2.2 Whole blood model*

One mL of whole blood was pipetted into twelve 2 mL Eppendorf tubes. Ten µL of (phosphate buffered saline) PBS was added to tubes #1-4. 10 µL 4.6 mmol/L GTS-21 working solution (final concentration approximately 45 µmol/L) to tubes #5-8; and 10 µL 9.2 mmol/L GTS-21 working solution (final concentration. approximately. 90 µmol/L) to tubes #9-12. The tubes were incubated at 37°C for 15 minutes on a rocking platform. Then, 10 µL PBS was added to tubes #1,2,5,6,9,10; 10 µL of the 1000 ng/mL LPS (final concentration 10 ng/mL) was added to tubes #3,7,11; and 10 µL 10000 ng/mL LPS (final concentration approximately. 100 ng/mL) was added to tubes #4,8,12. Thus, tubes #1-2 containing whole blood and PBS only, served as controls (Figure 12).
After 4 h incubation at 37° C on a rocking platform, plasma was collected by centrifugation (2600 g, 20 min, 18° C, Eppendorf centrifuge 5804R) and frozen at -80° C pending cytokine analyses.

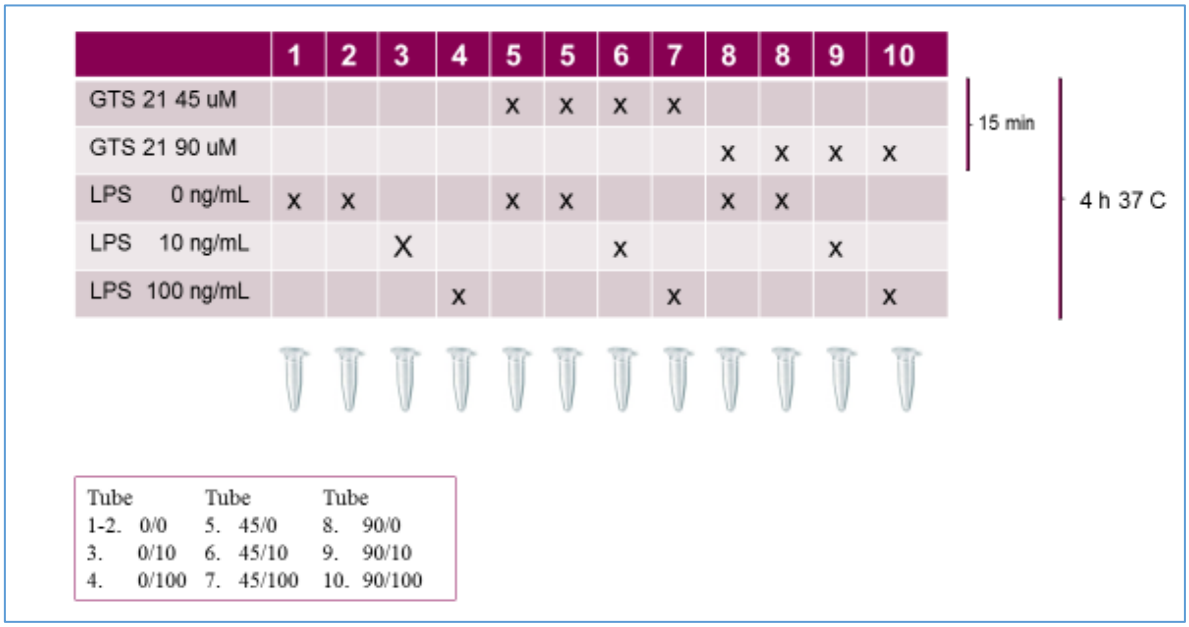



**Fig. 12.** *Schematic presentation of the whole blood assay with different concentrations of LPS and GTS 21.*

### 4.2.3 Cytokine analysis Study I-III

High sensitivity TNF, IL-1, IL 6 (Study I), and IL-10 were analyzed on an Immulite 1000 Analyzer from Siemens using immunometric assays (Siemens Healthcare Diagnostics, Los Angeles, CA, USA) according to the instructions of the manufacturers. PlFG was analyzed using ELISA kit from R&D Systems (R&D Systems Europe Ltd. Abingdon, UK).

#### *4.2.3.1 ELISA*

Enzyme-linked immunosorbent assay (ELISA) is a method for detecting and quantifying proteins, peptides, antibodies, and hormones. ELISA uses antigens, antibodies, enzyme-linked antibodies, and a solution with catalyzing substrate for activation of enzyme. There are two principle methods of ELISA; (169) in the *direct coating* method, the molecule of interest is diluted and directly attached to the inner surface of the wells of a microtiter plate by passive adsorption. In the *indirect coating* method, also called "capture" or sandwich ELISA, labeled antibodies or antigens precoats the wells. Serum, plasma, or cell culture supernatant with the molecule of interest is added to the wells and then unbound molecules are washed away. An enzyme-linked antibody is added, and in the last step, a solution with a reagent activates the enzyme which then produces fluorescent light. The intensity of the color quantifies the levels of the primary antibody or antigen. In study I-III the "sandwich" ELISA was used to quantify TNF, IL-1, IL-6, IL-10, and PlGF.

### 4.2.4 Routine blood samples Study I-III

High-sensitivity C-reactive protein (hsCRP), hemoglobin (Hb), white blood cell counts (WBC) with differentials, Creatinine, Potassium, and Sodium were analyzed by using routine methods at the Department of Clinical Chemistry at Karolinska University Hospital (study I and III) or at the Department of Clinical Chemistry at Södersjukhuset (Study II).

### 4.2.5 Heart rate variability Study I-III

HRV and thoracic bioimpedance (TBI) were recorded at the same time points as blood sampling (except in study I). ECG for HRV was recorded by using 3 gel coated electrodes positioned on the right hand, right leg and left leg. TBI was measured with 4 gel coated electrodes on the right and left side under the chest. The lower electrodes were used for current injection and the upper ones for voltage measurement. Before ECG recording patients performed one minute of controlled breathing (10-15 breaths per minute depending on patient comfort) to adjust for any incongruence in breathing pattern.
ECG and TBI were recorded for 20 minutes by using a custom-made device designed by Z-Health Technologies AB, Borås. The RR intervals variability were automatically calculated based on the Pan-Tompkins algorithm (170) implemented in Matlab 2015, MathWorks, Natick, Massachusetts, USA. The subsequent HRV analysis generated data for time and frequency domain parameters in accordance with European Task Force for HRV measurements (59). Time domain parameters e.g. SDNN were calculated as an overall

estimate of HRV and frequency domain were calculated with focus on LF, HF and LF/HF-ratio. TBI recordings were used to calculate the respiration rate and to check for respiratory sinus arrhythmia (RSA) as a source of difference in frequency analysis.

### 4.2.6 Renal denervation and coronary angiography Study II

#### *4.2.6.1 Renal denervation*

RDN was performed at the PCI unit at the Cardiology Department at Södersjukhuset, Stockholm. RDN patients received pre-treatment with diazepam and peri-procedural analgesia with an i.v. infusion of remifentanil. Antithrombotic treatment included Aspirin (a bolus dose of 320 mg at least 12 h prior to the procedure, followed by 75 mg o.d.) and a peri-procedure administration of a weight-adjusted dose (50-100 U/kg) of unfractionated Heparin (UFH) with a target activated clotting time (ACT) of 250 seconds.
Femoral artery access was established using standard 8 French (F) sheaths and guiding catheters were advanced to select left and right renal artery position. Radiofrequency ablation was performed bilaterally at a minimum of 6 locations in each renal artery using the EnligHTN™ multi-electrode renal denervation catheter (St Jude Medical, St Paul, MN, USA). In the presence of multiple renal artery anatomy, all branches with a diameter of > 4 mm were treated. For post-procedure hemostasis, an arterial closure device (8F Angio-Seal™, St Jude Medical, St Paul, MN, USA) and provisional manual compression was used. Following the procedure, patients were monitored in hospital for 24 h with pre-discharge evaluation of BP, arterial access site hemostasis and renal function.

#### *4.2.6.2 Coronary angiography*

CA was performed at the PCI unit at the Cardiology Department at Södersjukhuset, Stockholm. The procedure was performed with standard 6F sheaths and catheters through right radial artery access. All 4 disease controls received pretreatment with Aspirin (a bolus dose of 320 mg at least 12 h prior to the procedure, followed by 75 mg o.d.), paracetamol and diazepam. In the presence of coronary artery disease, a clinical decision on revascularization strategy was made. Standard procedure included implantation of drug eluting stents (DES) and the addition of antithrombotic treatment with a weight adjusted dose of low-molecular-weight-heparin (LMWH) as well as a second oral anti-platelet drug (Clopidogrel, loading dose of 600 mg followed by 75 mg o.d.). Post-procedure monitoring of BP, hemostasis and renal function were similar to that in RDN patients.

### 4.2.7 Minimally invasive oscillating device Study III

A Minimally invasive oscillating device developed for a rhinitis and a migraine study was used (171, 172). The VNS equipment consisted of a manually operated electric energy unit and a single-use plastic tube with an inflatable tip (Fig. 13a). The inflatable tip was made of thin latex and fixed at the end of the tube with a strong rubber band. Inside the latex tip there was a soft thin plastic pin anchored to the edge of the tube. The tip was lubricated with water

and manually inserted in the left nostril and a headband was used to secure the position of the tub and tip (Fig 13b).

a)  b) 

**Fig. 13** *The minimally invasive oscillation device. a) The catheter with inflatable tip b) The catheter inserted in left nostril and secured in position by special designed headband. Juto (171). Open access*

In study III the tip was inflated with air at a pressure of 95 mbar to fill out the nostril. In case of major deviation in nose cavity volume it was possible to adjust the size of the tip by moving the rubber band. A 25 mm soft pin inside the tip was set to oscillate at a frequency of 68 Hz. With the balloon in direct contact to the mucosa the study subjects sensed the oscillation as vibrations in their nose and forehead. Each treatment session lasted for 10 minutes. The choice of side, pressure, frequency, and standardized duration of treatment was made in discussion with the designer of the VNS equipment based on prior studies using the same device (171, 172).

### 4.2.8 Study IV

The HeartBEAT data was prepared as follows: ECG recordings were available from two timepoints, 318 at baseline and 301 at follow-up. Twenty-four ECG recordings from baseline and 32 ECG from follow-up were excluded due to short or noisy ECG within the target recording length (6±2 hours). Baseline and follow-up ECG were put together to a data set of 563 ECG-recordings. Each ECG recording was divided in 5, 10, 30, 60, 90, 120, 180, and 360 minutes epochs. Pre-processing was performed to remove powerline interference, reduce baseline wanders and high frequency noises by use of appropriate notch and bandpass filters. Processing and signal analysis were performed using custom scripts in MATLAB 2014a, (MathWorks Inc. Natick, Massachusetts, USA). R-peaks were detected and RR intervals calculated by using the Pan-Tompkins method (173).

HRV analysis was conducted using a selection of conventional HRV indices in the time domain, frequency domains, and the non-linear methods described by the Taskforce for HRV measurement and analysis (174). HRV indices were calculated for ECG epochs of 5, 10, 30, 60, 90, 120, 180, and 360 minutes.

Correlation between HRV indices and inflammatory markers (CRP, TNF, IL-6, E-Selectin) were computed by Pearson correlation coefficient controlling for potential confounding factors. Both inflammatory markers and skewed HRV indices (SDNN, SDANN, RMSSD,

HR, LF, HF, VLF, SD1, and SD2) were transformed by natural logarithm (ln) to attain normal distribution. This calculation was repeated for all 5, 10, 20, 30, 60, 120, 180, and 360 minutes epochs.

The prediction value of HR and HRV indices (SDNN, SD2) in combination with individual characteristics (age, gender, BMI and betablocker) was calculated in a hierarchical 7-level model using multiple regression and least squares regression. Level 1 used only the mean HR, level 2 added the SDNN, level 3 added the non-linear index SD2, and levels 4-7 included the individual characteristics; age, gender, BMI, and beta-blocker, respectively.

For detection of differences between HRV comparing patients with CRP < 5 mg/mL and CRP ≥ 5 µg/mL an independent two-sample student t-test was used. To compensate for an unbalanced distribution (more cases with CRP <5 µg/mL) a weighted least squares regression was used by giving higher weights to values with less probability.

### 4.2.9 Statistics

#### *4.2.9.1 Study I*

Comparisons between groups were performed using non-parametric Wilcoxon's test. Comparisons between groups and various concentrations of LPS were performed using Friedman's non-parametric two-way ANOVA. Fischer's exact test and chi-square tests were used for categorical variables. Non-parametric Spearman's rank correlation analysis was used to determine associations between various variables.

#### *4.2.9.2 Study II*

Difference between two groups were assessed with the non-parametric Wilcoxon test for continuous variables and Chi-square test (Fischer's exact test) for ordinal/nominal variables. Comparisons between more than two groups were assessed with non-parametric ANOVA Kruskal-Wallis test for continuous variables. We performed no adjustments for multiple comparisons i.e. Bonferroni procedures.

#### *4.2.9.3 Study III*

Comparisons between four time periods were assessed with nonparametric analysis of variance Kruskal -Wallis ANOVA test.

Statistical analysis in study I-III were performed using statistical software SAS version 9.4 (SAS Campus Drive, Cary, NC, USA).

#### *4.2.9.4 Study IV*

See description of method in chapter 3.2.8. Analyses were performed using SPSS Statistics 23 (IBM Inc., New York, USA) following the Leard Statistics guidelines (175).

# 5 RESULTS

## 5.1 STUDY I – THE CHOLINERGIC ANTI-INFLAMMATORY PATHWAY ACTIVITY IN DIALYSIS PATIENTS: A ROLE FOR IMMUNOMODULATION?

A total twenty patients with CKD 5 and RRT were included. The cohort comprised twelve patients (7 male and 5 female, age range 26-84 years) on HD treatment and eight patients (5 male and 3 female, age range 47-84 years) on PD treatment. Patients in HD were treated 3 times per week on average 4 hours per dialysis session, whereas PD patients were treated with standard CAPD regimen except for two who used APD. Eight healthy controls were included (5 male and 3 female, age range 31-52 years).There was a significant difference in age between patients and controls, but not in gender.

### 5.1.1 Whole blood assay and routine blood samples

CRP, TNF, IL-1, Il-6, and IL-10 were significantly increased at baseline in unstimulated blood samples in patients compared with controls ($p=0.004$, $p=0001$, $p=0.02$, p-0.004, $p=0.002$ respectively). After LPS stimulation with the concentrations of 10 and 100 ng/mL there was an increase of TNF and IL-6 levels though, significantly stronger in patients at maximal stimulation compared with controls ($p<0.01$). IL-1 and IL-10 was significantly increased in patients at both concentrations of LPS compared with controls ($p<0.01$). WBC and differentials did not differ between groups.

In the presence of GTS-21 there was a strong decrease of TNF and IL-1 in patients that did not differ significantly from controls, but a statistical difference remained between groups with higher levels in patients ($p<0.01$). The same pattern for IL-6 was observed however without differing between patients and controls. IL-10 increased in the presence of GTS 21 in a dose-dependent manner, but only in patients.

### 5.1.2 HRV

HRV and TBI were recorded in a subset of the dialysis patients (7 in HD and 5 in PD) and in all 8 healthy controls. For practical reasons these recordings were not done at blood sampling but at a separate visit. Patients and controls were assessed in a quiet examining room and in a supine position. There were no significant differences for HRV indices between the HD and PD patients. The result of the HRV analysis in patients confirmed significant autonomic dysfunction compared to controls regarding time domain parameter SDNN, as well as the frequency domain parameters HF and LF. Respiration rate and heart rate did not differ between groups.

## 5.2 STUDY II – THE CHOLINERGIC ANTI-INFLAMMATORY PATHWAY IN RESISTANT HYPERTENSION TREATED WITH RENAL DENERVATION

Ten patients with resistant hypertension were recruited as suitable for RDN (7 male and 3 female, median age 62 years, age range 47-71 years). Four patients (3 male and 1 female, median age 66 years, age range 59-76 years) scheduled for elective coronary angiography due to ischemic heart disease served as DC.

### 5.2.1 Whole blood assay and routine blood sample

DC had significantly higher TNF and IL-1 than patients at baseline (p=0.009 and p=0.03 respectively) but there was no difference in IL-10 levels.

One day after RDN/CA the basal cytokine levels were unchanged in DC whereas there was a decrease of TNF in RDN-patients (p<0.03). LPS-stimulated levels of pro-inflammatory TNF and IL-1 were significantly lower 1 day after RDN (LPS 0, 10 and 100 ng/mL TNF; p=0.0009, p=0.0009, p=0.001, IL-1; p=0.0001, p=0.002, p=0.005 respectively). Anti-inflammatory IL-10 levels were significantly higher on day 1 (p=n.s, p=0.02, p=0.01 respectively). However, at 3 and 6 months the LPS-stimulated cytokine levels were similar to levels before RDN in the incubated 4 h samples. The stimulated cytokine levels in DC did not differ before CA and at day 1 after the procedure.

In the presence of 90 μmol/L GTS-21 at day 1 after RDN, the TNF levels in LPS-stimulated blood exhibited an even more marked reduction (p<0.001), but these TNF levels also weakened during follow-up (Fig. 14).

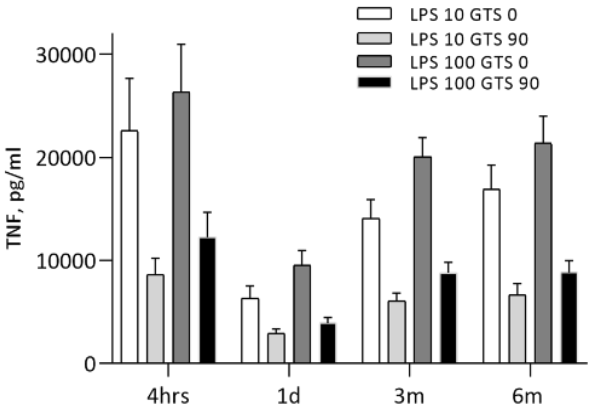



**Fig. 14.** *Levels of TNF in the LPS-model with LPS-concentrations 10 and 100 ng/mL and GTS 21 in concentrations 45 and 90 μmol/L. The effect of RDN (LPS 10+0 and LPS 100+0) was most pronounced one day after RDN but declined with time at follow up. The addition of GTS 21 (LPS 10 + GTS 90 and LPS 100 + GTS 90) resulted further reduction of TNF. Hilderman (176). Reprinted with permission from Oxford University Press.*

### 5.2.2 HRV

HRV indices (SDNN, RMSSD, HR, HF, LF/HF) did not show any significant changes in RDN patients on day1, or at 3 and 6 months. RDN patients however had lower HRV indices

as compared to norms established by the Task Force of the European Society of Cardiology and the North American Society of Pacing and Electrophysiology 1996, suggesting underlying autonomic dysfunction (59).

### 5.2.3 Placental growth factor

Baseline PlGF was similar in DC and RDN patients, but higher than in healthy controls. PlGF was subsequently measured at all time points in RDN patients but did not differ during follow-up.

### 5.2.4 Blood pressure

DBP was significantly lower one day after renal denervation (p=0.001) (Fig. 15). No further significant differences in BP were found.

| Patients (n=12) | |
|---|---|
| Sex female (%) | 38 |
| Age (years) | 60 (49-82) |
| Vintage dialysis (months) | 96 (16-233) |
| P-hsCRP (mg/mL) | 6 (0.4-44.6) |
| B-Hb (g/L) | 106 (97-113) |
| B-WBC (x109) | 6.4 (4.8-10.2) |
| B-Monocytes (x109) | 00.7 (0.4-1.0) |
| B-Lymfocytes (x109) | 1.4 (0.8-1.9) |
| S-TNF (pg/mL) | 20.3 (14.2-77.4)* |
| S-IL-1 (pg/mL) | 1.3 (0.3-9.2)* |
| S-IL-10 (pg/mL) | 1.2 (0.8-2.6)* |
| Medications, patients | |
| B-blocker | 6 |
| Alfa-blocker | 1 |
| Cal channel antagonist | 5 |
| ACE/ARB- inhibitor | 6 |
| Furosemide | 4 |
| Statins | 3 |

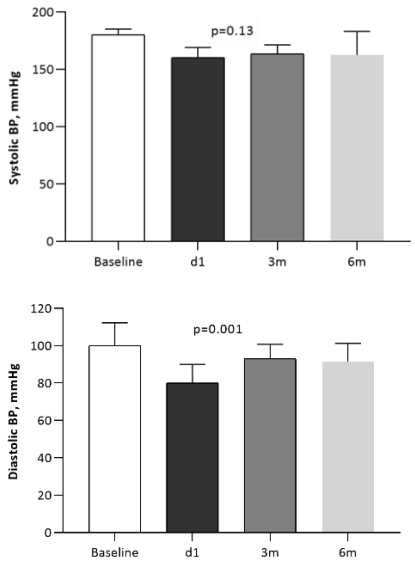



**Fig 15** *SBP and DBP at all time points.*

*Additional figure not published in paper. Hilderman.(177)*

## 5.3 STUDY III – MINIMALLY INVASIVE VAGUS NERVE STIMULATION IN HEMODIALYSIS PATIENTS AND INFLAMMATORY RESPONSE

Twelve HD patients were enrolled consecutively between November 2014 and November 2015 (8 male, 5 females; age range 47-86 years). See table 2 for clinical characteristics, blood chemistry, and medication. The primary kidney diseases are shown below in figure 16. All patients completed the 4 weeks protocolized intervention period, but 4 of the patients were affected by either infection or access surgery during follow-up. The intervention procedure was generally well tolerated except minor problems such as sneezing and lacrimal tear flow. There were no major adverse events during the intervention period.

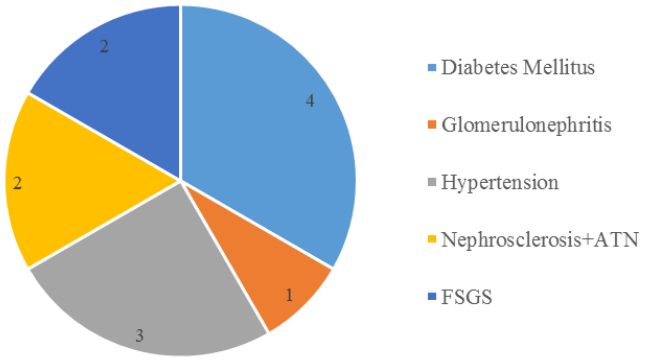



**Fig. 16** *Underlying diseases, study III.*

### 5.3.1 Whole blood assay and routine blood samples

The levels of CRP and unstimulated cytokines did not change significantly during the study although there was a trend towards decreased CRP (ANOVA, p=0.61) and IL-1 (ANOVA, p=0.63) as well as increased IL-10 (ANOVA, p=0.37). In the whole blood assay, we saw a small trend of TNF and IL-1 reduction and increase of IL-10, during the intervention period however again without reaching statistical significance. In the presence of the cholinergic analogue, GTS 21, there was a further approximately 50% reduction of cytokine release. Here shown as TNF (Figure 17). We also analyzed the 8 patients that completed the study and follow-up without any hurdles separately. Although the TNF decrease was slightly more pronounced during treatment and follow-up, the results again did not reach statistical significance (Fig. 17). The trend for Il-1 was similar as for TNF, but the IL-10 tendency was not as clear.

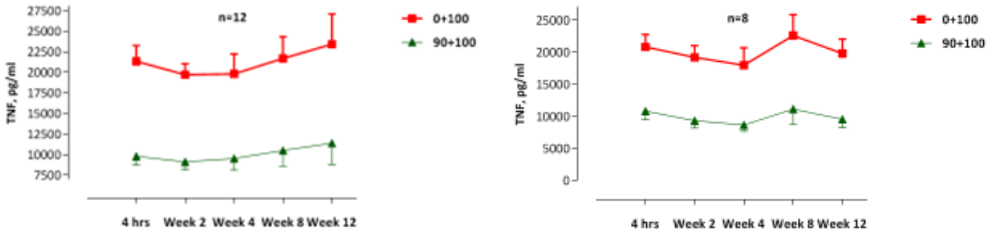



**Fig. 17** *Levels of TNF without (red) and with (green) addition of GTS 21, in the LPS whole blood model. N=12 and n= 8.*

### 5.3.2 HRV

#### *5.3.2.1 HRV in healthy control*

Calculation of HRV variables in a healthy volunteer from ECG recording simultaneous with 10 minutes of VNS treatment, using the Minimally invasive oscillating device, showed an increase of time domain variables SDNN (47.7 - 61.0 ms), RMSSD (34 - 41.5 ms) as well as frequency domain variables LF (559 - 1749 $ms^2$ and HF (255 - 382 $ms^2$) suggesting an influence on vagal tone (Fig. 18 a,b)

**Fig. 18 a)**

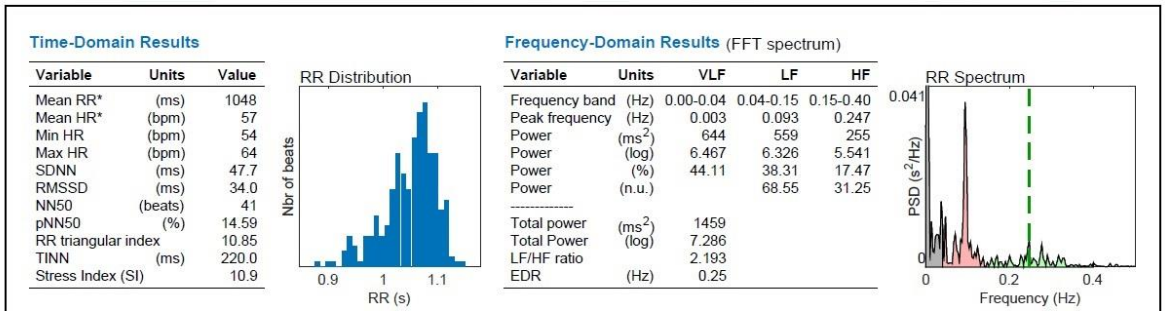



**Fig. 18 b)**

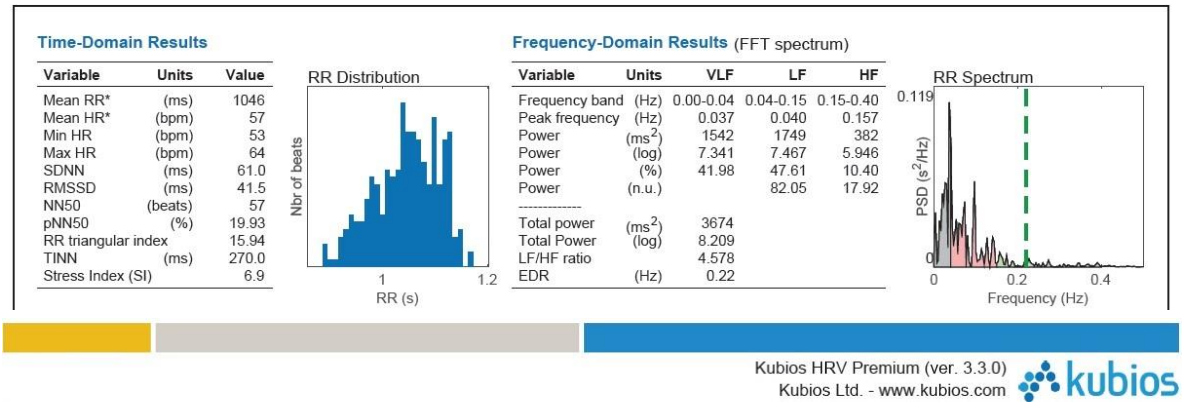



**Fig.18** *HRV before (a) and after (b) treatment in a healthy volunteer, top and bottom results respectively. Time domain RMSSD and SDNN are increased after 10 minutes treatment with as well as frequency domain LF and HF (b).*

#### *5.3.2.2 HRV in hemodialysis patients*

Three patients had a cardiac pacemaker or an implantable cardioverter-defibrillator (ICD) and were thus not analyzed for HRV indices. In none of the remaining 9 patients did HRV analysis show any significant changes during the study or follow-up.

Outside of the protocol 3 out of 4 patients with insulin dependent diabetes mellitus reported a lowering of insulin-doses with about 25% during the study. The fourth patient had only a small dose (4 E) long-acting insulin at night. Approximately 3 months after finishing the study patients had resumed their original insulin dosing.

## 5.4 STUDY IV – PROGNOSTIC POTENTIAL OF HEART RATE AND HEART RATE VARIABILITY FOR MONITORING THE CHOLINERGIC ANTI-INFLAMMATORY PATHWAY

### 5.4.1 Correlation of HRV and inflammatory cytokines

CRP, IL-6, TNF, and E-Selectin had a significantly positive correlation with HR and between each other. There was a negative correlation between SDNN and IL-1, IL-6, and E-selectin. SD2 was also negatively correlated to E-Selectin. After controlling for confounding factors (age, gender, beta-blocker, BMI), the correlations remained significant at p=0.05.

### 5.4.2 Multiple regressions using least mean squares

Level 7, which included mean HR, SDNN, SD 2, age, gender, BMI, and betablocker resulted in the highest correlation coefficient and coefficient of determination for inflammatory markers. Level 7 showed the highest predictive power for CRP while TNF showed the least correlation with multiple regression (Fig. 19).

### 5.4.3 Between group comparisons

In comparison between groups (CRP <5 and CRP ≥5 µg/ml) regarding HR and HRV indices calculated from 5-360 minutes epochs, a positive correlation was observed for CRP ≥5 µg/ml and HR. SDNN was negatively related to CRP and hence patients with higher CRP levels had lower SDNN. However, this difference was not significant for shorter epochs i.e., 5-60 minutes, although the trend of lower SDNN for CRP ≥5 µg/ml levels was consistent. SDANN was also significantly lower for CRP ≥5 µg/ml but only in 5-minute epochs. Frequency domain measurements i.e. HF, LF, and LF/HF seemed to be less reliable and were occasionally lower or higher between groups. SD2, which is a non-linear long-term measurement based on the Poincaré plot, seemed to be consistently lower, occasionally significant for the group with higher CRP levels. Figure 19 illustrates that the 7$^{th}$ level (including all parameters) that showed the highest specificity.

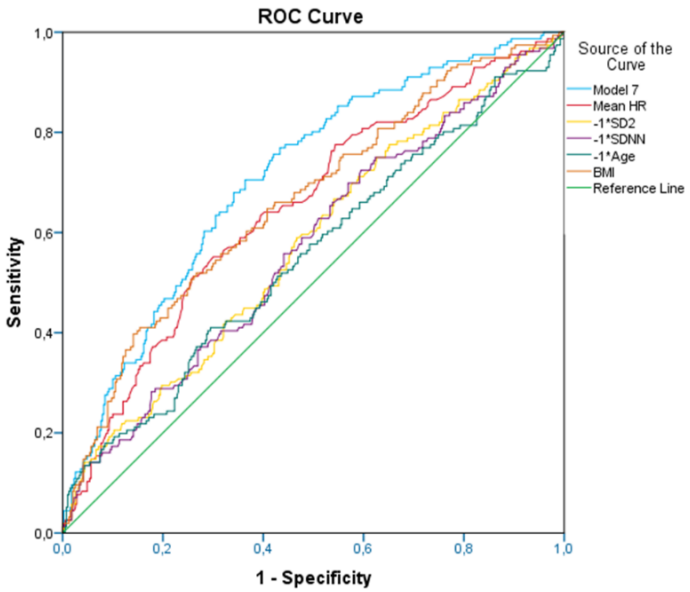



**Fig. 19** *ROC analysis for mean RR interval, Ln SDNN, Ln SD2, BMI, age and linear regression in group with CRP >5 µg/mL. A combination of personal characteristics and HRV indices improves the specificity for predicting CRP ≥ 5 µg/mL. Level 7 result in a sensitivity of 83.3% for predicting cases with CRP ≥5 µg/mL however, with a specificity of 30.7%.*

# 6 DISCUSSION

The Cholinergic Anti-inflammatory Pathway is a neuro-immune circuit that safeguards the homeostatic balance of inflammatory activity in response to injury, pathogens, and tissue ischemia. The afferent portion of the parasympathetic vagus nerve deliver the bulk of sensory

information from the thoracic cavity and subdiaphragmatic organs and tissues. The motor or the efferent arm of this so-called inflammatory reflex, subsequently responds to and regulates the inflammatory response. Several pre-clinical studies have established how this important link between the nervous system and the immune system communicates and interacts (26, 33, 135, 178, 179).

The recent understanding that inflammation and excessive sympathetic activity can be a significant contributor to, or even be causing, non-communicable chronic diseases that represent the greater part of the global burden of disease (CVD, stroke, cancer, and pulmonary disease), has placed neuro-immunological science and the novel concept of bioelectric medicine in the spotlight.

Chronic inflammation is highly associated with CDK, ESRD and hypertension and is linked to a significant increased morbidity and mortality rate (13, 87, 180, 181).

In this thesis we have studied the CAP and autonomic dysfunction in patients with ESRD, and in patients with resistant hypertension treated with RDN. Our main findings are that ESRD patients seem to have a functional CAP, with regard to LPS-stimulated cytokine release in a whole blood model, and that RND treatment has an immediate impact on CAP potentially by influencing CAP bur not with long-lasting effects (176, 177). Furthermore, we gained new insights in the possible use of VNS in dialysis as well as the optimal duration of HRV measurement and potential correlates with inflammatory cytokines.

*Study I*

The finding in study I, with regard to elevated baseline levels of pro-inflammatory cytokines in dialysis patients, corroborates findings from previous studies and reflect an underlying status of chronic inflammation (87, 91, 92, 98, 99, 176, 180-183). A negative correlation between kidney function, expressed as GFR, and inflammatory cytokines and markers (IL-1, IL-6, TNF, CRP, and fibrinogen) is well established, but may in part be a retention phenomenon due to a decreased clearance of these substances (96, 181). However, elevated circulating pro-inflammatory cytokines levels have repeatedly been associated with atherosclerosis, CVD, and increased mortality (10, 87, 89, 90, 97, 98). Contributory factors, besides inflammation per se, are compromised immune responses due to uremia and an impaired performance of neutrophils and lymphocytes. Factors that, all in all, contribute to an increased susceptibility and an amplified risk of infections (87, 184-188).

The LPS-whole blood model has previously been used in studies of RA patients (176, 189). We could however note that the dialysis patients diverged from the RA patients by having a much more pronounced inflammatory response to LPS in the whole blood model (176, 189, 190). The reason for this may be that the RA patients were treated with anti-inflammatory drugs, which was not the case with the dialysis patients, or that there is a possible intrinsic difference between RA and CKD regarding mounting an inflammatory response. However, in both groups, where there was a distinct autonomic dysfunction, the pattern of response to

the cholinergic agonist was similar, suggesting a functional CAP irrespective of underlying disease.

*Study II*

In this study we found a significant decrease in baseline TNF levels one day after RDN, but not in the disease controls. We also found strong and significant changes for all 3 cytokines (TNF, IL-1b, and IL-10) in the *ex vivo* LPS stimulated whole blood model, implying that cytokine modulation is an instantaneous event after RDN. Yet none of these changes remained after 3 and 6 months. This is the first study that has examined anti-inflammatory effects longitudinally in the short-, medium, - and long-term after RDN. However, Zaldivia *et al.* demonstrated that RDN reduces cytokine markers, monocyte activation, and monocyte-platelet aggregate formation at 3 and 6 months follow up (191). Our results and the ones by Zaldivia, the latter including a larger number of patients, however, indicate that the effect may not be long-lasting or possibly weaker with time. This is of relevance, not only due to the renewed interest in RDN as a therapy for resistant hypertension, but also for other potential long-term effects. Furthermore, there is in our study no evidence showing, that interventions altering sympathetic activity through RDN and thereby potentially changing the balance in the autonomic system, can modulate HRV. Nevertheless, this probably should be addressed in future studies with larger cohorts. The same may be suggested for inflammation and PlGF, the latter being a possible marker of a potentially modifiable neuro-immunological pathway in the spleen that may contribute to hypertension (109).

Interestingly, the cholinergic analogue GTS-21, strengthened the magnitude of TNF suppression compared to RDN alone, suggesting a possible mechanistic pathway and potential for an additional anti-inflammatory effect in resistant hypertension.

A role for monocytes in hypertension was investigated in a genetic mouse model of LysM+ monocytes depletion leading to a blunted angiotensin-II–induced hypertension. By adoptive transfer of proinflammatory monocytes the BP elevation was reestablished, which may serve as evidence of the importance of monocytes in hypertension in this model (118).

A role for α7nAChR in hypertension has been deduced from a model of α7nAChR deficient mice exposed to hypertension, where increased end organ damage and higher pro-inflammatory cytokine levels in serum was found. Chronic administration of PNU-282987, a selective α7nAChR agonist, reduced tissue levels of TNF, IL-1, and IL-6 and likewise decreased end organ damage in spontaneously hypertensive mice, highlighting the therapeutic potential of α7nAChR agonists for reducing hypertension-associated morbidity (192).

Taking everything into account, these findings suggest that repeated or long-term use or stimulation of CAP or α7nAChR agonists may be interesting to explore for future therapeutic interventions in hypertension.

*Study III*

Recent human studies, based on previous animal studies, have shown that electrical or electromagnetic energy via cervical implants stimulating the vagus nerve may be beneficial for patients with inflammatory diseases such as RA and IBD (151, 153, 154, 193, 194). Furthermore, in addition to DAS28-CRP, a disease score used in RA, the LPS induced inflammation whole blood model, as well as and other inflammatory markers, have been used as outcome parameters in studies of VNS in RA (190).

Chronic inflammation is a typical finding in CKD and in dialysis patients and is linked to CVD and all-cause mortality, not only in adult but also in pediatric CKD patients (195). However, few trials have been conducted to address this medical conundrum. This is mainly because CKD and dialysis patients are a complicated patient group for inclusion in clinical studies. A high number of co-morbidities as well as an excessively high susceptibility for infections in addition to expected, as well as unexpected, complications such as dialysis access problems, have been perceived as impediments (196).

In this study we used a minimally invasive VNS device rather than an implantable device to treat 12 hemodialysis patients before each dialysis session for 4 weeks. The baseline levels of CRP and cytokines did not change significantly during this small pilot study, although visually there was a trend towards a decreased CRP, TNF, and IL-1 as well as augmented IL-10. In the LPS induced whole blood model we saw a small trend of TNF and IL-1 reduction and IL-10 increase during intervention. In the presence of the cholinergic analogue GTS 21 there was an additional 50 % reduction of cytokine (TNF) levels.

We used an equipment that generated vibrations in the left nostril. It may be questioned whether vibrations per se are adequate for an afferent vagus nerve signal to be initiated. Nevertheless, in a study by Addorisio *et al.* a “vibrotactile” device was utilized to propagate vibrations to the cymba choncha of the outer ear. Both healthy subjects and patients were given treatment which resulted in decreased levels of circulating cytokines in healthy subjects and lower TNF and disease activity and in patients (164). As the vagus nerve has sensory fibers in the pharynx mucosa it is therefore reasonable to assume that the vibrations from the VNS equipment we used could have a similar effect (197).

Although our study did not lead to significant changes in inflammatory markers it may still serve as a first step to potentially utilize VNS in this patient group. The enhanced reduction of cytokine levels, after adding GTS-21 in the whole blood model, suggests that there may be a further potential for cholinergic modulation in patients. Prolonged treatment time at each session or a longer protocolized treatment period could be the target for a new and larger trial, especially if novel non-invasive devices are proven effective.

As the spleen is essential for CAP activation recent studies have investigated local modulation of splenic activity. In studies by Giglotti *et al.* a modified ultrasound regimen to the spleen was demonstrated in animal models of acute kidney disease and that ultrasound may have a protective effect on AKI in sepsis (198). It was also confirmed that the beneficial

effect was dependent on α7nAChR on immune cells and not α7nAChR in the parenchyma (199).

Yet another aspect of VNS treatment was elucidated in an experimental intestine inflammation model. An implanted VNS electrode was attached to the abdominal part of the vagus nerve and stimulation resulted in lower CRP, normal stool, and improved histology. VNS did not alter HRV parameters in this study but HR declined during the VNS procedure (200).

In another potential application for kidney patients, Hoeger *et al.*, have interestingly shown that vagal stimulation in brain dead donor rats had the ability to decrease chronic allograft nephropathy in recipients. This would be an example of CAP contributing to adoptive transfer of immune cell and thereby protection against trauma caused by surgery as well as inflammation due to rejection (201).

A very intriguing finding was noted outside the protocol. Three out of four patients with diabetes (both T1DM and T2DM) could reduce their insulin doses with 25 % during the study. The fourth patient had only a small dose (4 E) long-acting insulin at night. This is in line with the results in the study by Hanes *et al.*, in which galantamine was given to patients with metabolic syndrome (165). Galantamine treatment for 12 weeks was shown to ameliorate inflammation as well as lowering plasma insulin and insulin resistance as compared to placebo. These results suggest that the association between inflammation and insulin resistance is a potential treatment target.

Other examples of effects observed outside the protocol was better sleep among several of the participants. This was manifested by a reduction of the need of sleeping medication. Furthermore, several subjects reported an improvement in general mood as well as alertness. We cannot however exclude that these effects could be secondary to the attention given to patients during the study and the VNS treatment. During the VNS study we utilized questionnaires such as SF 36 and EQ5D, but unfortunately these do not include detailed questions to describe more subjective experiences and mood changes. These questionnaires are more directed toward physical status and activities in daily life and may have been to blunt to capture these items as well as cognitive function. For future VNS studies these quality of life items, as well as other Patient-Reported Outcome Measures (PROM) and Patient-Reported Experience Measures (PREM). may be important to assess.

*Study IV*

The significant correlation between HR and HRV and pro-inflammatory cytokines in this study are consistent with previous findings (62, 202-204). In addition, the between group comparisons indicate that patients with higher CRP levels (CRP ≥5 μg/mL) have a higher HR independent of the number of averaged heart beats and hence length of ECG recording. In contrast, SDNN showed a significant difference associated with longer recordings. In this study, we could not replicate previously reported significant correlations between frequency

domain variables and pro-inflammatory markers except for E-Selectin (62, 205, 206). However, these previous studies were performed on healthy adults and with different circumstances of ECG-recordings; 10 minutes sitting (206), 24 hour Holter ECG (205), and 11 minutes sitting (62).

Malik et al. have described that LF and HF in combination with HR might be used to estimate basal vagal tone (207). Resting HR in healthy subjects, as a measure of autonomic balance, was compared with individuals at risk of RA and with RA patients. Autonomic dysfunction was not found in healthy subjects but in individuals at risk of RA. This was was confirmed in an independent cohort, where elevated resting HR was associated with arthritis development over time (152). A lower HR in combination with high HRV (SDNN) could be interpreted as higher vagal tone in patients with lower CRP. Indeed, high HR and low HRV are signs of low vagal tone. The prognostic value of heart rate can be observed from correlations, the equivalence of the created 7-level model in this study, and from the ROC analysis. It can be hypothesized from the 7-level model that using personal characteristics in addition to HRV will improve the prognostic value of CRP. Moreover, Houston *et al*. suggest that HR and HRV alone have the potential to be used as a tool to identify patients who may benefit from VNS (63). HR and HRV has been used in several studies to reflect the degree of neural modulation obtained from VNS (208-211) or pharmacological stimulation (212). Analysis of HRV collected by emerging wearable technologies e.g. smart watches would make it possible to study lager cohorts and detect early alterations in HRV. Combining HRV data with personal characteristics in longitudinal studies may potentially uncover lifestyle risk factors. Thus, HRV may potentially be used in a broader context of "personalized health".

## Limitations

*Study I*

The limitation of this study was the relatively small sample size of both patients and controls, and moreover that patients and controls were sampled only once. Nevertheless, in a study of RA patients of similar design, patients came for 1-8 visits. There were neither significant differences in whole blood assay results nor HRV findings at repeated visits suggesting that the phenotype in the individual patient is quite similar(189). Another limitation is that ECG-recordings for HRV were not performed in all patients and not at the same time as blood sampling, the latter for practical reasons. All healthy controls were monitored for HRV, but similarly as for patients, neither of them at the same occasion as blood draws. Another issue was that the control population was significantly younger compared to dialysis patients, which could be important as HRV is known to decrease with age (59, 213).

Lastly, it can also be claimed that the *ex vivo* whole blood model with GTS-21 does not fully represent the *in vivo* biology. Extensive experience with this model still indicates that the cells producing TNF are monocytes responsive to α7nAChR mediated signaling (190).

Furthermore, GTS-21 has been proven effective as an immunomodulatory compound by attenuating proinflammatory cytokine levels and improving survival in sepsis models (63, 143).

*Study II*

The limitations of study II were several. The use of RDN was interrupted following the neutral results of SYMPLICITY HTN-3 trial, which limited the number of included patients. Moreover, the control group was not a sham RDN group although they underwent an invasive procedure involving larger arteries. Furthermore, only office blood pressure was measured at baseline and follow-up at 6 months. Ambulatory blood pressure monitoring is commonly used in clinical trials of RDN and is considered to more adequately capture the variability of estimates of blood pressure changes (214). Finally, we considered whether the use of pre- and periprocedural administration of i.v. diazepam and analgesia could have had some anti-inflammatory effect per se in the RDN patients, but the true influence of this may been difficult to assess (215).

*Study III*

In this pilot study we recruited only 12 patients which may have influenced the study outcome to some extent. During follow-up four out of twelve patients suffered from events associated with inflammation, which added to the difficulties in interpreting the results. Another important factor was a quite wide range of baseline levels of inflammatory markers. The VNS device used in the study had not previously been applied in a study focusing on inflammatory response, and thus may not have been effective enough. However, a more likely explanation for the minor results is probably the fact that we only had one device and could not deliver the treatment daily which would have been preferable. Previous clinical studies, investigating VNS in inflammatory conditions, have had used protocols with implantable devices allowing daily and, in some cases, repeated daily sessions of stimulation. An implantable VNS device in dialysis patients could however, in our view, have been problematic in a patient population with a ubiquitous risk of infectious complications. For future studies, we aspire to more frequent and prolonged VNS treatment, but preferably using non-invasive easy to administer devices.

*Study IV*

This was the study with the largest number of patients. Still there were some shortcomings. In this study an absolute threshold of a CRP level of 5 µg/mL was used for comparison between groups. In healthy individuals a CRP threshold of 3 µg/mL is suggested to be the range for intermediate to high risk factors for developing coronary heart disease (216, 217). However, a threshold of 3 µg/mL in this study would probably not have changed the results significantly, regarding correlation with HRV, but may have had some influence on the threshold level where this correlation is valid.

Circadian variation of HRV and HR and altered HRV and HR during apnea, has been reported (218). However, in our analysis the apnea episodes were not excluded but considered as strong short-term confounding factors. The absence of additional anti-inflammatory cytokines in the HeartBEAT database was a limitation that should be considered in further studies.

# 7 CONCLUSION

Study I

We have here shown that immune cells from dialysis patients can mount an anti-inflammatory response after cholinergic stimulation. These are novel findings in this patient population afflicted with autonomic dysfunction coupled with an underlying dysregulated cytokine response. In the evolving therapeutic field of neuroimmunomodulation and bioelectronic medicine our data is a piece of the puzzle, which may facilitate future trials using cholinergic modalities in the CKD population.

Study II

Hypertension contributes significantly to cardiovascular morbidity and mortality rates worldwide. Catheter-based radiofrequency ablation has been used as an invasive add-on treatment for resistant hypertension in clinical trials. We have shown that modulation of renal sympathetic nerve activity trough RDN has an instantaneous effect in a model of LPS-stimulated cytokine release, but that is not sustained over time. When adding a cholinergic analogue to LPS-stimulated samples, a more marked effect on cytokine levels was recorded. This may indicate that repeated or long-term stimulation of CAP, rather than a single RDN treatment, could potentially be more effective in reducing inflammation. The axis involving sympathetic-parasympathetic nerves, PlGF, and inflammatory cells as a potential target for therapeutic intervention by neuro-modulating devices in hypertension merits further research.

Study III

Although the reduction of inflammatory markers did not reach statistical significance in this short-term VNS pilot study, we suggest that our study could serve as a foundation to potentially utilizing VNS in this patient group. One may speculate that prolonged daily treatment time at each session or a longer protocolized treatment period could have improved the outcome as was the case in recent clinical trials. Studies using cervical vagus implants have demonstrated significant inhibition of cytokine production and disease activity in patients with RA after 4 to 8 weeks with daily VNS and 6 months of daily VNS in Crohn´s disease. However, a non-invasive device for self-use would be preferable to reduce the burden of chronic inflammation in CKD patients prior to and on dialysis.

Study IV

HRV is the most common tool to measure the activity of sympathetic and parasympathetic nerve activity. HRV is known to correlate with inflammatory markers which was shown also in this study. The selection of markers, however, was limited. Longer ECG recordings (360 min) was shown to reflect SDNN the best. For other variables it seems that shorter recording are as reliable as longer ones. Regarding HRV the optimal duration of HRV measurements and potential correlates with inflammatory cytokines should be studied further in clinical trials.

# 8 FUTURE PERSPECTIVES

The concept of the immune system and the nervous system as being two different entities without any interaction is no longer true. There is uncontested evidence of communication and collaboration, and furthermore that the immune system is a prerequisite for the function of the nervous system and vice-versa.

Recent and ongoing research further expands this perspective and future therapeutic possibilities. Both CKD and hypertension are associated with chronic inflammation as are many other major non-communicable diseases. HRV analysis and the development of other physiological assessment methods may become more useful in clinical practice in the future. Muscular sympathetic nerve activity has been shown to be elevated in patients with EHT both when untreated and during treatment with antihypertensive drugs. (106). This may suggest a previously unrecognized masked sympathetic activation and baroreflex dysfunction in true resistant hypertension. Newer non-drug related devices for treatment of hypertension are under development (219, 220). In the future it may be important to determine which patients could benefit from this kind of intervention (221, 222). The potential use of tools that may more effectively address both hypertension and inflammation is of considerable interest.

There are also ongoing efforts to find biological markers for HRV as a substitute for HRV. A recent study has tried to evaluate serum cholinesterase activity (CAA) in healthy subjects to estimate autonomic balance. CAA at rest was shown to predict some of the HRV parameters changes during exercise in a sex-dependent manner. Even more interesting was that a correlation was found between higher CAA and detectable high sensitive Troponin T (hsTnT) in women, in which hsTnT is normally not detected without known cardiovascular disease (223). Whether this can be used as an early risk marker for cardiovascular disease warrants further studies.

Increased morbidity and mortality in CKD are continuous major problems. Some of the underlying causes and disease mechanisms are well known. These factors are still a challenge to address on a population basis, but also in the individual patient. Better dialysis treatment and better tools to reduce chronic inflammation is certainly of outmost importance, whereas pre-dialysis patients need other types of interventions which among other components should also focus on inflammation. CKD with or without dialysis and associated co-morbidities contribute to years of decreased quality of life for patients. Its share of both national and international health economy is furthermore substantial. Interesting new interventional studies in the field of CAP in AKI, and CKD, which also involves our preliminary results from the VNS in dialysis study, may in the future be of great and substantial benefit in AKI, AKD, CKD, and associated inflammatory conditions.

# 9 POPULÄRVETENSKAPLIG SAMMANFATTNING

**Bakgrund**: Det icke viljestyrda, autonoma nervsystemet, består av sympatikus som förbereder kroppen för kamp och flykt och parasympatikus som är mer aktivt i vila. Obalans mellan sympatisk och parasympatisk aktivitet, autonom dysfunktion, kan mätas med EKG. Utifrån EKG beräknas variationer i tid mellan hjärtslagen – hjärtslagsvariabilitet (HRV). Autonom dysfunktion och kronisk inflammation ses vid en rad olika sjukdomar, till exempel reumatism (RA), inflammatorisk tarmsjukdom (IBD), diabetes, högt blodtryck och kronisk njursvikt. Både autonom dysfunktion och inflammation är förknippade med ökad sjuklighet och dödlighet i framförallt hjärt- och kärlsjukdom hos dessa patienter.
Den kolinergiska anti-inflammatoriska effektorvägen (CAP) är en nervkrets där vagusnerven reglerar det medfödda immunförsvaret. Vid tex vävnadsskada, syrebrist eller bakterieangrepp bildar immunförsvaret ämnen (cytokiner) som en del av den inflammatoriska reaktionen. Vagusnerven reagerar på detta och sänder signaler till förlängda märgen som reflexmässigt skickar signaler till mjälten. I mjälten finns celler som tillverkar en substans, acetylkolin, som när den når det inflammerade området dämpar immunförsvarets aktivitet. På så sätt kan kroppen kontrollera och reglera den inflammatoriska aktiviteten och förhindra att den blir för hög så att kroppen tar skada istället för att läka.
I små studier på patienter med RA och IBD har man stimulerat vagusnerven (VNS) med en pacemakerliknande anordning och sett positiva effekter.
**Syftet** med studierna i denna avhandling var att undersöka om dialyspatienter har immunceller som reagerar på stimulering av vagusnerven och om det skulle kunna påverka nivåerna av cytokiner i blodet. Vi ville också undersöka patienter som genomgått renal denervering (RDN) (”bränning” av sympatiska nerver i njurens artärer) pga. svårbehandlat högt blodtryck. Vår frågeställning var om de skulle få minskade nivåer av cytokiner som resultat av minskad sympatisk nervaktivitet. Slutligen var syftet med avhandlingen också att utforska förhållandet mellan cytokiner och olika långa mätningar av hjärtslagsvariabilitet.
**Material och metoder**: I våra studier har vi använt blodprov som stimulerats med LPS (del av bakterievägg) för att åstadkomma en kraftig inflammatorisk reaktion. När cytokinnivåerna stigit har vi satt till ett ämne (GTS 21) som liknar det som mjälten utsöndrar och mätt om cytokinnivåerna, dvs inflammationen minskade. I studie I lämnade 20 dialyspatienter och 12 friska kontroller blodprov. I studie II tog vi blodprov på 10 patienter som behandlades med RDN och följde dem i sex månader. I studie III lät vi 12 dialyspatienter genomgå VNS före varje dialys i fyra veckor (12 behandlingar). Stimuleringen gjordes med en liten vibrerande ballong i vänster näsborre i 10 minuter. Blodprov togs vid flera tillfällen under och efter behandlingsperioden. I alla studierna tog vi EKG på försökspersonerna och beräknade HRV. I studie IV använde vi en databas med nattliga EKG-mätningar för att titta på samband mellan hjärtslagsvariabilitet och cytokiner.
**Resultat**: I studie I såg vi att dialyspatienter producerar cytokiner i högre utsträckning än friska men när vi tillsatte GTS 21 i proverna sjönk cytokinnivåerna lika mycket i bägge grupperna. I studie II visade blodproverna att RDN-patienter hade lägre cytokinnivåer dagen efter RDN men efter 6 månader hade de stigit till utgångsnivån igen. I studie III kunde vi inte

se klart minskade cytokinnivåer under eller efter VNS-behandling även om det fanns en trend. Studie IV visade att 360 minuters EKG registrering var mest rättvisande avseende vissa HRV-mått och att det fanns ett visst samband med cytokinnivåer.
**Slutsats**: Dialyspatienter har en fungerande CAP och VNS skulle kunna vara en möjlig behandling för att minska inflammation och potentiellt risken för hjärt-och kärlsjukdom. Troligen behövs dock intensivare behandling med dagliga eller längre behandlingar. RDN minskar cytokinnivåerna på kort sikt men är i sin nuvarande form inget alternativ som antiinflammatorisk behandling. I framtiden kan långtidsmätningar av HVR möjligen användas i kliniska studier på inflammation som ett mått på behandlingseffekt.

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